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(54) Titre : IDENTIFICATION DE BACTERIES PATHOGENES
(54) Title: DETECTION OF PATHOGENIC BACTERIA

(57) Abrégé/Abstract:
The invention relates to oligonucleotides, which can be used to detect pathogenic bacteria. Said oligonucleotides assist in a method which enables pathogenic bacteria to be selected from non-pathogenic bacteria. The detection of the bacteria preferably includes a polymer chain reaction (PCR). The invention also relates to oligonucleotides, which can be used as a positive test for the PCR.

Abstract

The object of the invention are oligonucleotides which can be used for the detection of pathogenic bacteria. With the aid of these oligonucleotides a method can be applied which enables the selection of pathogenic bacteria from non-pathogenic bacteria. Preferably the detection of the bacteria includes a PCR. Oligonucleotides are also provided which can be used as a positive control for the PCR.

CERTIFIED TRANSLATION FROM GERMAN

Detection of pathogenic bacteria

This invention relates to a method for the detection of EHEC bacteria and to oligonucleotides suitable for this detection.

In the age of international transport and rational processing methods the importance of pathogenic bacteria transmitted through foodstuffs is growing. Often raw materials from many different parts of the country are brought together at a central point, mixed thoroughly and processed to form a certain foodstuff. If one of the raw products was the carrier of a pathogenic germ, then it can reproduce during the production process and lead to the contamination of a large batch of foodstuff.

In this connection *Escherichia coli* has arisen as a very important pathogenic germ. Following campylobacter and salmonella, it is the third most common germ contaminating foodstuffs. The bacterium normally occurs as a harmless commensal in the human intestine. However, it can take up certain pathogenicity genes and can then represent a fatal risk. Consequently, a whole series of *E. coli* sub-types have been characterised which have high pathogenic potential. These include the Shigella strains which are really to be grouped systematically under *E. coli*. Also worth mentioning are EPEC (enteropathogenic *E. coli*) which in particular cause diarrhoea illnesses with newborn/infants, ETEC (enterotoxinogenic *E. coli*), which form extracellular thermally stable and thermally unstable toxins and are mainly responsible for travelling diarrhoea and EIEC, which penetrate the cells of the intestinal mucosa and cause bacillary dysentery.

An especially dangerous group of pathogenic *E. coli* strains are the EHECs (enterohemorrhagic *E. coli*). The group of EHECs also includes the particularly frequently occurring serotype O157:H7. This, as also the other members of the group,

can cause the haemolytic-uraemic syndrome (HUS) which can be fatal. HUS is accompanied by diarrhoea containing blood and acute kidney failure.

The endemic occurrence of EHECs in nature is largely restricted to cattle, even if other sources, in particular pigs, have been documented as reservoirs. As a consequence, processed beef products, in particular minced meat, are often contaminated with EHECs. In some investigations into foodstuffs more than 50% of minced meat samples were positive to EHEC. In recent years other foodstuffs such as lettuce, radishes, milk and milk products have been identified as EHEC sources.

In the USA in the last few decades more than 20,000 *E. coli* O157:H7 infections occurred per annum (Boyce et al. 1995, N. Engl. J. Med. 333, 364-368), of which about 250 ended in death. However, the real figures may be much higher due to defective diagnosis. In Europe and Japan *E. coli* O157:H7 infections are primarily reported in summer. In contrast, in the southern hemisphere non-O157 EHEC serotypes are in particular of great importance.

The pathogenic potential of an EHEC strain is determined by its pathogenicity factors. Consequently, the occurrence of Slt genes (Shiga-like toxin or vtx = verotoxin gene) is a necessary, but not a sufficient prerequisite for pathogenicity. In addition, other factors have been characterised (Nataro and Kaper 1998, Clin. Microb. Rev. 11, 142-201), which are necessary to infect the host. Many of these factors are not constantly coded in the genome, but are rather located on transferable plasmides or in phage genomes. Therefore, the equipping of EHEC strains with pathogenicity factors may also be subject to chronological variability.

The reliable diagnostic detection of EHEC strains with known methods causes substantial problems. So microbiological methods are hardly suitable for obtaining reliable detection. Metabolic physiological differences between apathogenic *E. coli* and pathogenic EHEC strains are hardly present. The frequently characteristic defect of the uidA gene (beta-glucuronidase) for *E. coli* O157:H7 (Cebula et al. 1995, J. Clin. Microb.

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33, 248-250) is not a reliable feature of the EHEC group. For this reason diagnostic methods must fall back on molecular biological features.

One of the methods frequently used in the past was serotyping by an ELISA. However, this presents many disadvantages, because it is relatively time-consuming and demands many working steps. In addition, its sensitivity is not sufficient for many diagnostic applications. Furthermore, the serotype alone is not a sufficient feature for pathogenicity.

Another method of differentiating between *E. coli* strains is to investigate differences in the DNA sequence. The technique is based in particular on the fact that pathogenic strains possess certain toxin genes. For example, the toxin genes similar to Shiga (Shiga-like toxins, slt or verotoxin genes, vtx) could be directly detected (Takeshi et al. 1997, Microb. Immun. 41, 819-822, Paton and Paton 1999, J. Clin. Microb. 37, 3362-3365). The PCR can be applied to amplify parts of the gene. These fragments can be rendered visible so that they act as a diagnostic characteristic.

The disadvantage of this method is that the slt genes are not a sufficient prerequisite for pathogenicity. Other DNA sequence features are necessary to establish an unambiguous correlation between the genotype and pathogenicity. The *E. coli* strains, which possess slt genes are designated VTECs (verotoxin forming *E. coli* or STECs). Consequently, they form a larger group than the EHECs.

Other genetic markers for EHEC or subgroups of it have also been tried out. These include the fimA gene (Li et al. 1997, Mol. Cell. Probes, 11, 397-406) and the fliC gene (Fields et al. 1997, J. Clin. Microb. 35, 1056-1070). However, they all have the disadvantage of mapping only part of the EHEC group.

Since the EHEC group does not form a systematic unit phylogenetically, there arises the difficult task of finding genetic polymorphisms through which it is unambiguously characterised. These polymorphisms should also be so reliable that they also acquire

heterogeneities and genetic instabilities within the EHEC group. Apart from the specific detection, they should also permit the most sensitive detection of EHEC possible.

There are already some detection systems for *E. coli* classified as EHECs. Where they are based on immunological detection, their sensitivity is however not sufficient. In addition the detection of antibodies is very sensitive to external contaminations. Extracts from foodstuffs present significant problems, because they conceal the antigen surfaces of the bacteria or even destroy them. Where though some surface antigens reach exposure, they are often too few to ensure reliable detection with adequate sensitivity.

The object of this invention is to provide a method which ensures the reliable detection of EHEC bacteria in any sample and which is subject to the lowest possible impairment due to other constituents of the sample, such as PCR inhibitors, the DNA of non-pathogenic bacteria, or due to the quenching phenomenon (refer to the chapter "Optimisation of the on-line PCR"). Also, the object of the invention is to make the means required for EHEC detection available.

The first problem is solved according to the invention by a method for the detection of EHEC bacteria, incorporating the step of detection of the occurrence of a nucleic acid sequence from the Slt locus and/or eae locus and/or hlyA locus in the sample.

The second problem is solved according to the invention by an oligonucleotide selected from one of the nucleic acids including at least one sequence with one of the SEQ ID numbers 1 – 98 and/or derivatives of it.

Definitions

Fragments of oligonucleotides

Fragments of oligonucleotides arise due to deletion of one or more nucleotides on the 5' and/or 3' end of an oligonucleotide.

Gene

The gene includes the open reading frame or coding area of a DNA. Also, the cistron is a gene which together with other cistrons is however located on one mRNA. DNA regions which regulate the transcriptions of the gene, such as the promoter, terminator, enhancer also belong to the gene.

Identical DNA sequences / percentage of identity

For the determination of the identity (in the sense of complete matching, corresponding to 100% identity) of DNA or RNA sequences, partial sequences of a larger polynucleotide are considered. These partial sequences comprise ten nucleotides and are then identical when all 10 modules are identical for two comparative sequences. The nucleotides thymidine and uridine are identical. As partial sequences, all possible fragments of a larger polynucleotide can be considered.

As an example two polynucleotides are considered which comprise 20 nucleotides and which differ in the 5th module. In a sequence comparison six 10-way nucleotides are found which are identical and five which are not identical, because they differ in one module.

In addition, the identity can be gradually determined, whereby the unit is stated in percent. For the determination of the degree of identity partial sequences are also considered, which comprise as a minimum the length of the actually used sequence, e.g. as primer, or 20 nucleotides.

As an example, polynucleotide A with a length of 100 nucleotides and B with a length of 200 nucleotides are compared. A primer with a length of 14 nucleotides is derived from polynucleotide B. For the determination of the degree of identity, polynucleotide A is compared with the primer over its complete length. If the sequence of the primer occurs in polynucleotide A, whereby it however deviates in one module, then there is a fragment with a degree of identity of 13:14 → 92.3%.

In the second example the polynucleotides A and B previously mentioned are compared in their entirety. In this case all the possible comparative windows of a length of 20 nucleotides are applied and the degree of identity determined for them. If then nucleotides nos. 50-69 of polynucleotide A and B are identical with the exception of nucleotide no. 55, then a degree of identity of 19:20 → 95% arises for these fragments.

Multiplex PCR

A multiplex PCR is a Polymerase Chain Reaction or DNA or RNA amplification reaction in which more than two primers are used which are not regarded as a forwards-backwards primer pair. With the presence of all nucleotide target molecules to be detected, this leads to the creation of at least two different amplicons. These amplicons should at least differ in the region in which the primers link, but they can also be allocated to completely different genes. In the case of detection of the EHEC, the multiplex PCR, in the simultaneous detection of two or three genes, consists of the group SltI, SltII, eae and hlyA.

Nucleotides

Nucleotides are the modules of the DNA or RNA. The following abbreviations are used:

G = Guanosine, A = Adenosine, T = Thymidine, C = Cytidine, R = G or A, Y = C or T, K = G or T, W = A or T, S = C or G, M = A or C, B = C, G or T, D = A, G or T, H = A, C or T, V = A, C or G, N = A, C, G or T, I = Inosine.

On-line detection

In relation to this invention, on-line detection is defined as the simultaneous running of two processes: the detection of the DNA or RNA and a process which leads to the provision of a detectable amount of DNA or RNA. With this process the release of genomic DNA/RNA from cells may, for example, be involved or the enrichment of DNA/RNA from a complex mixture or the amplification of polynucleotides, e.g. through a PCR. Detection is the perception of a signal which correlates to the presence and possibly the amount of the DNA/RNA. In the case of the PCR this type of signal may increase with the increasing amplification of the target DNA. On-line detection can be carried out also in a miniaturised form, e.g. on a chip. The signal can, for example, be produced through the fluorescent molecules of a probe, through radioactive molecules or through enzyme-coupled colour or fluorescence intensity.

The term on-line detection is synonymous to real-time detection.

Primer

Primers are oligonucleotides which act as starter molecules during a PCR. Here, they hybridise on a target molecule, which may be, for example, DNA or RNA, and are lengthened by a polymerase. They can also however act as probes.

Probe

Probes are oligonucleotides which hybridise on the target DNA or RNA molecules. They are used for the direct or indirect detection of these target DNA or RNA molecules. For this purpose, they can be coupled to fluorescent molecules or to molecules containing colouring agents. In addition they can be indirectly detected with an ELISA. In a special version they only produce a signal through FRET (Fluorescence Resonance Energy Transfer) when two probes hybridise adjacently in a defined manner. In this case a colouring agent on a probe is excited by a light beam and transfers its excitation energy to the colouring agent of the adjacent probe. This then emits light of a defined wavelength. They can also be used as primers.

EHEC and VTEC

EHECs are enterohemorrhagic *E. coli* and a subgroup of the VTEC. *E. coli* of the serotype O157 is a subgroup of the EHEC.

VTEC is characterised in that it either possesses the Sltl (vtx1) or the Sltll (vtx2) or both genes. EHECs are VTECs which also possess the eae gene and/or hlyA gene (coded for Intimine). In addition, they can be characterised by the presence of other pathogenicity genes such as hlyB, hlyC, fimA, fliC, etc.

Slt locus

Slt locus signifies the locus containing the Sltl gene or Sltll gene, which are also designated as vtxl resp. vtxll. The nucleic acid sequence of this locus is known from the state of the art, for example from Paton, A.W. et al. 1995, Gene 153 (1), 71-74. The

term "locus" as used in this connection comprises, apart from the coded region, also a section of 1000 nucleotides in each case on the 5' end of the start codon or on the 3' end of the stop codon.

eae locus and hlyA locus

The sequences of the eae locus and the hlyA locus are also known from the state of the art, for example from Makino, K., et al. 1998, DNA Res. 5 (1), 1-9.

Derivatives of the oligonucleotides according to the invention

Derivatives of the oligonucleotides according to the invention are taken to mean sequences which differ in at least one nucleotide from the specific sequences according to SEQ ID numbers 1 – 98, for example, by at least one base interchange, an insertion, deletion or addition. These also include oligonucleotides which are at least 80% identical to one of the specific sequences according to SEQ ID numbers 1 – 98 and oligonucleotides with a comparable specificity of hybridisation. The latter signifies that the derivative produces the same hybridisation pattern with a specified sample containing nucleic acid, such as the oligonucleotide with one of the specific sequences with one of the SEQ ID numbers 1 – 98.

Biochip

Biochip is taken to mean carriers for the high throughput of analyses as marketed, for example, by AFFYMETRIX. The chips enable the testing of numerous different nucleic acids on one carrier.

The analysis of DNA exhibits substantial advantages compared to the serological detection, because there are standardised, simple purification methods for DNA analysis with which DNA can be separated from external matrices and purified further. Due to the size of the bacterial genome, selection can also take place from a substantial number of individual sequence motifs, whereas the selection of the previously mentioned exposed surface antigens is relatively low.

As sequences for the specific detection of EHEC bacteria, sequences from the Slt locus, the eae locus and the hlyA locus are suitable. Here, it is sufficient for the detection of EHEC in a specified sample if a partial sequence from the Slt locus and another of the quoted loci can be detected in the analysis sample. With the Slt locus two different gene loci are actually involved, SltI and SltII, whereby however only one of the two loci occurs with the numerous EHEC strains. The simultaneous detection of sequences from the Slt locus and the eae locus in a single sample provides sufficiently high proof. The simultaneous detection of a sequence from the Slt locus and the hlyA locus has a similar high reliability. A particularly high degree of reliability with regard to an EHEC contamination then arises if sequences from the three different loci, Slt, eae and hlyA, are simultaneously detected in one sample.

With another preferred embodiment the nucleic acid to be examined is passed to a PCR. This has the result that EHEC-specific amplicons are produced if nucleic acids of EHEC bacteria are present in the sample. Here in the simplest case, the PCR can be arranged as a simple linear PCR with only one oligonucleotide as primer, but preferably the PCR takes place however with so-called forwards and backwards primers for each genome section of the bacterial nucleic acid to be amplified.

With another preferred embodiment a primer combination is used whereby at least one primer is selected, comprising at least one sequence from one of the SEQ ID numbers 1 – 45 and 95 – 98, also designated as sequences of the categories A – C and a primer, comprising at least one sequence selected from one of the SEQ ID numbers 46 – 83 and 93 and 94, also designated as sequences of the categories D and E. According to

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the invention, derivatives of the mentioned primers can also be used for the detection. The derivatives normally lead to amplification of the same genome sections as indicated by the definitive primers according to the SEQ ID numbers 1 – 98.

With another preferred embodiment a primer pair consisting of a forwards primer and a backwards primer, selected from the category A – C, is used with a primer pair comprising a forwards primer and a backwards primer, selected from the category D and E. A preferred embodiment uses a primer pair from one of the categories A – C in combination with a primer pair from category D and another primer pair from category E.

With a further preferred embodiment the detection method includes the use of another primer comprising at least one sequence, selected from a sequence from category F. These sequences are characteristic of the genus *E. coli*. Consequently, for example, with a preferred strategy of EHEC detection, the analysis sample can be first analysed with a sequence selected from the category F. A positive result points to the presence of *E. coli* in the analysis sample. In a second step it can then be more closely determined, using the sequences from the categories A – E, whether the detected *E. coli* is a member of the EHEC group. The additional analysis with sequences from the category F can also occur of course as an additional measure after the analysis with the sequences from the categories A – E.

With a further preferred embodiment the various oligonucleotides and therefore the various PCR runs are carried out in the form of a multiplex PCR. Here, different amplicons are created in the PCR in a single initiated reaction with the aid of the various oligonucleotides. Alternatively, the multiplex PCR can also be subdivided to different PCRs, whereby a sequential train of PCRs is carried out, whereby each PCR is carried out with a specific primer or primer pair. In both cases, with the presence of EHEC bacteria in the analysis sample a band pattern is obtained indicating the presence of EHEC bacteria.

With a further preferred embodiment use is made of the so-called chip technology (biochips) in the detection method. Here, on one hand a large number of different analysis samples can be analysed on one chip in that the individual spots on the chip contain analysis material from different sources. On the other hand, the chip can carry a set of oligonucleotides, whereby each spot contains a specific oligonucleotide and this oligonucleotide pattern is brought into contact with analysis samples. In the case that the analysis material contains EHEC nucleic acid, it hybridises with the probes specific to the EHEC present on the chip and produces a corresponding signal pattern.

With a further preferred embodiment the detection method can include further steps, such as for example an amplification of the nucleic acid to be detected, whereby this preferably occurs using PCR and/or a southern hybridisation with EHEC-specific probes, whereby this hybridisation occurs without prior amplification or after amplification of the nucleic acid to be detected is concluded. Furthermore, the nucleic acid to be detected can be detected using the ligase chain reaction. Finally, the nucleic acid to be detected can be enriched by isothermal nucleic acid amplification.

With a further preferred embodiment, the amplification of the target nucleic acid can also take place using on-line detection.

With a further preferred embodiment the amplification of the nucleic acid to be detected and/or the detection of the contained amplicons occurs on a biochip, whereby it is particularly preferable to carry out the amplification and detection on one chip.

According to the invention, as a means for carrying out the method described above, oligonucleotides are selected from a nucleic acid, comprising at least one sequence with one of the SEQ ID numbers 1 – 98 or derivatives thereof. The stated oligonucleotides can on one hand be used as primers within the scope of a PCR and on the other hand also as probes, for example within the scope of a southern blot hybridisation. Depending on the requirements of the desired detection, the specialist can form the suitable combination of oligonucleotides as primers or probes.

With an especially preferred embodiment a combination of oligonucleotides is used, whereby at least one oligonucleotide is selected from sequences from the categories A – C and at least one oligonucleotide is selected from sequences from the categories D and E.

With another especially preferred embodiment the combination according to the invention furthermore comprises an oligonucleotide selected from the sequences of category F which are specific to the genus *E. coli*. Preferably, the stated oligonucleotides or combinations of them are used in the form of a kit for the detection of EHEC bacteria, whereby the kit also includes other reagents for the detection of bacteria or for conducting the detection reactions. In this respect, the reagents and enzymes required for the PCR and, where applicable, suitable carrier materials are also included, for example, such as is desired with the chip technology.

The oligonucleotides or oligonucleotide combinations according to the invention are therefore a suitable means for the specific and reliable detection of EHEC bacteria in any analysis samples.

With the invention of the polymerase chain reaction it is possible to amplify individual DNA polynucleotides and then to detect them with extremely high sensitivity. This technology opens up substantial new opportunities, but also exhibits new problems. For example, with the DNA amplification incorrect fragments can be easily amplified, leading to incorrect positive results in the analysis. In addition, it is very difficult to select the diagnostic DNA sequences characteristic to EHEC from the multitude of possibilities.

Bacteria enrichment culture

↓

DNA/RNA release, purification

↓

Amplification & on-line detection

Flowchart for the detection of EHEC by PCR and simultaneous detection

This invention consists of a method and oligonucleotides which enable a qualitative and quantitative detection of EHEC. This method also includes a positive check for the PCR reaction which detects the genera of *E. coli* and Shigella. This is important, because with negative EHEC findings the correct sequence of the PCR reaction must be ensured. The detection method consists all together of four steps: propagation of the bacteria, purification of the DNA/RNA, amplification of the polynucleotides and detection of them. In a special method the two last steps can also take place simultaneously.

The propagation of the bacteria occurs in that the matrix to be investigated, e.g. a foodstuff or faecal sample is incubated with a currently available bacterial medium. Bacterial media are commercially available and can, for example, contain a proteolytically digested basic substance, such as soya broth, bile salts and a buffer such as dipotassium hydrogen phosphate. In addition, it is advantageous to add an inhibitor to the enriching medium which promotes the growth of the EHEC compared to other bacteria in the enrichment medium. Such inhibitors may be antibiotics, such as Novobiocin, for example.

In the second step the polynucleotides are purified. To do this, the bacteria are normally first separated from the medium by centrifuging and/or filtration. A further washing stage may follow. Then the bacteria are broken down. This takes place by heating, by an alkaline or acidic environment or by reagents which destabilise the bacteria cell wall,

such as deionising chemicals or lysozyme. The genomic DNA or the RNA can now be directly used in a PCR reaction or it is purified further. For this purification materials are suitable on the surface of which the polynucleotides bond, e.g. positively charged surfaces or silicate surfaces. This material can be mounted in columns and is commercially available.

The PCR reaction and the detection of the amplicons represent the greatest importance in the detection of bacteria. As already explained, it is very difficult to find differences in DNA sequences between EHEC and other bacteria, in particular the harmless *E. coli* strains. A single PCR reaction with the amplification of a single DNA or RNA region alone would not appear to offer a very reliable foundation for marking the strain limits. A preferred element of the invention is that various regions of the EHEC genome can be amplified simultaneously and/or sequentially. Preferably, further DNA/RNA sequences are amplified in a consecutive step for the concluding analysis. If all significant amplicons can be detected simultaneously, e.g. on one chip, then the "first" amplification step and the "consecutive" amplification step can also run in a single PCR reaction or in a single PCR reaction vessel. The key to the application of the primers and probes is given below.

The system for the detection of EHEC makes primers available which optimally map the EHEC group in certain combinations. The detection is, for example, carried out in two independent PCR runs in primer multiplex arrangements. In a first run the primers and probes of categories A, B and/or C are employed. In the second run only the samples are used which were positive in the first run. In this second run the primers and probes of categories D and E are used. Within one category a forwards primer and a backwards primer can be combined with one another in each case. So multiplex PCRs are carried out in which many target DNA or RNA fragments are propagated simultaneously in one reaction. Due to this process a very differentiated picture of the bacterial populations present can be obtained. Depending on the practical requirements to the sensitivity of the EHEC detection and when the simultaneous detection is

possible, all detection primers (for categories A+B+C and D or E and possibly category F) can also be used in a single multiplex PCR.

Tab. 1: Forwards primers, category A

No.	Primer sequence
1	CTGGGGAAGGTTGAGTAG
2	GTCCTGCCTGAYTATCATGG
3	ACAAGACTCTGTTCGTGTAGG
4	AAGAATTTCTTTTGRAAGYRTTAATGC
5	AATTCTGGGWAGCGTGGCATTAATACTG

Tab. 2: Backwards primers, category A

No.	Primer sequence
6	CCCACTTTAACTGTAAAGGT
7	CGTCATCATTATATTTTGTATACTCCACC
8	CACTTGCTGAAAAAAATGAAAG

Tab. 3: Probes, category A

No.	Probe sequence	Probe pair
9	AGCGTGGCATTAATACTGAATTGTCA	1
10	ATCATGCATCGCGAGTTGCCAGAAT	1
11	GTCCTGCCTGAMTATCATGGACAAGACTCT	2
12	TTCGTGTWGGAAGAATTTCTTTTGRAAGYRTTAAT	2
13	ATGAGTTTCCTTCTATGTGYCCGGYAGATGGAA	3
14	TCCGTGGGATTACGCACAATAAAATATTTGTGGGATT	3
15	AAAYATTATTAATAGCTGCATCRCTTTCATTT	4
16	TTCAGCAAGTGYGCTGGCKRCGCCWGATTCTGTA	4, 5
17	ACTGGRAAGGTGGAGTATACAAAATATAATGAT	5
95	ATTAAYRCTTYCAAAAGAAATTCTTCC	6
96	CAGTATTAATGCCACGCTWCCCAGAATT	6
97	CCTTCTATGTGYCCGGYAGATGGAA	7
98	TSCGTGGGATTACGCACAAT	7

Tab. 4: Forwards primers, category B

No.	Primer sequence
18	GGCACTGTCTGAAACTGCT
19	GAAACTGCTCCTGTKTATAC
20	GATGACRCCGGRAGAMGTG
21	CTGAACTGGGGGMGAATCAGCAATGTG

Tab. 5: Backwards primers, category B

No.	Primer sequence
22	YGCCATTGCATTAACAGA
23	GCWGCKGTATTACTTTCCCATAA
24	GGCCTGTCGCCAGTTATCTGACATTCTGGTTG
25	TCTCTTCATTCACGGCGCG

Tab. 6: Category C, forwards primers

No.	Primer sequence
26	GGCGCTGTCTGAGGCATCT
27	GAGGCATCTCCGCTTTATAC
28	AATGACGGCTCAGGATGTT
29	CTGAACTGGGGAAGAATAAGTAATGTT

Tab. 7: Category C, backwards primers

No.	Primer sequence
30	GCAGCGATTGTATTCGCTTCCCACAAAACA
31	GCCCTGTCTCCAACAATCTGGCATTCTGTTTT
32	CTGTTTTTGGCTCACGGAACG
33	CGCCATGGAATTAGCAGAAAAG

Tab. 8: Probes, category B

No.	Probe sequence	Probe pair
34	CCCCAGTTCAGWGTGAGGTCC	1
35	CCGGAAGCACATTGCTGATTC	1
36	GAATATCCTTTAATAATATATCAGCGATACTKGG	2
37	WGTGGCSGTTATACTGAATTGYCATCATCAGGG	2
38	CGTTCYGTTCGCKCCGTGAATGAAGAKA	3
39	CAACCAGAATGTCAGATAACTGGCGACAGGCC	3

Tab. 9: Probes, category C

No.	Probe sequence	Probe pair
40	CCCCAGTTCAGGGTAAGGTCA	1
41	CTGGAAGAACATTACTTATTC	1
42	AGGATATCTTTTAATAGTCTTTCTGCGATTCTCGG	2
43	TGTTGCGGTCATCCTTAATTGCCACTCAACCGG	2
44	TTATTCAGTTCGTTCCGTGAGCCAAAAAC	3
45	AAAACAGAATGCCAGATTGTTGGAGACAGGGC	3

Tab. 10: Category D, forwards primers

No.	Primer sequence
46	CATGCTGCITTTTTTAGAAGA
47	CATGCTGCRTTTTTTAGAAGA
48	CATGCTGCITTTTTTAGAAGACTCT
49	CATGCTGCRTTTTTTAGAAGACTCT
50	AATGAATGGGAAAAGGAGCATGGC
51	CTCTCTGTCTTTGCTTGCTGATT
52	CTCGTCAGCATGCAGTAGAAAGAGCAGTCG
53	CATTGGGATGAGAAGATCGGTGAACTTGCAGG

Tab. 11: Category D, backwards primers

No.	Primer sequence
54	CGTCTTTATCTCCGAGYTCAG
55	ACATCGTCTTTATCTCCGAGYTCAG
56	TTTACCAACATCCGTCTTATTATAAGATACGG
57	CCTTCACCAGCAAATACTTCTG
58	TGAGCCTGCTCCAGAATAAACC
59	TCAATTTTGAATAATCATATACA

Tab. 12: Probes, category D

No.	Probe sequence	Probe pair
60	AGAGAAAGAAAACAGAGTGGTAAATATGAATATATGACAT	1
61	TCTTATTGTAAATGGTAAGGATACATGGTCTGTAAAAG	1
62	GGGACCATAGACCTTTCAACAGGTAATGTATCAAGTGTTTT	2
63	ACATTTATAACACCAACATTTACCCCAGGAGAAGAAG	2
64	GGCATATATTAATTATCTGGAAAATGGAGGGCTTTTAGAGGC	3
65	CAACCGAAGGAGTTTACACAACAAGTGTTTGATCCTC	3
66	CATTGGGATGAGAAGATCGGTGAACTTGCAGGCAT	4
67	AACCCGTAATGCTGATCGCAGTCAGAGTGGTAAGGC	4

Tab. 13: Category E, forwards primers

No.	Primer sequence
68	GGCCTGGTTACAACATTATGG
69	ACGCGAAAGATACCGCTCTTGGTAT
70	CCAGGCTTCGTCACAGTTGCA
71	GGAACGGCAGAGGTTAATCTGCAG
72	AGTGGTAATAACTTTGACGGTAGTTC

Tab. 14: Category E, backwards primers

No.	Primer sequence
73	ATCCCCATCGTCACCAGA
74	AACATTATCACCATAATACTG
75	TAGTTTACACCAACGGTCGCCGC
76	CATTACCCGTACCATGACGGT
77	CGGAACTGCATTGAGTAAAGGAGATCA

Tab. 15: Probes, category E

No.	Probe sequence	Probe pair
78	TCCAGTGAACTACCGTCAAAGTTATYACCAC	1
79	CCAGCATKTTTTCGGAATCATAGAACGGTAATAAGAA	1
80	ATGTTGGGCTATAACGTCTTCATTGATC	2
81	AGGATTTTTCTGGTGATAATACCCGT	2
82	AGGTATTGGTGGCGAATACTGGCGAGACTATTTCAAAAGT AG	3
83	TTAACGGCTATTTCCGCATGAGCGGCTGGCATGAGTCAT AC	3
93	TCCAGTGAACTACCGTCAAAGTTATYACCAC	4
94	CCAGCATKTTTTCGGAATCATAGAACGGTAATAAGAA	4

In addition to the detection of the EHEC, it is advantageous to control the correct sequence of the method. This invention ensures this control in that it enables the genus-specific detection of *E. coli*. Especially, a differentiation with respect to enterobacteria, such as the genus citrobacter, is advantageous, because in many cases these bacteria have accepted pathogenicity genes from *E. coli* in a horizontal gene transfer. An incorrect positive classification as VTEC can therefore be avoided.

Since *E. coli* and Shigella form one unit from a molecular biological point of view and also in many taxonomical classifications, these two genera are not separated during the control. This is very practicable in practice, because in microbiological routine diagnostics differentiation between these genera does not normally take place.

Tabs. 16+17 contain primers which enable the detection of *E. coli* and Shigella. For the investigation, aliquots of the same DNA/RNA samples can be used as for the EHEC detection. In addition, it is possible to carry out the *E. coli* control reaction simultaneously, i.e. in a reaction vessel together with the EHEC detection or in parallel. Furthermore, the *E. coli* / Shigella detection is also suitable for differentiating these genera from others.

Tab. 16: Category F, forwards primers

No.	Primer sequence
84	CGG GTC AGG TAA TTG CAC AGT A
85	CGG GTC AGG TGA TTG CAC AGT A
86	CGG GTC AGG TGA TTG CAC AAT A
87	CGG GTC AGG TAA TTG CAC AAT A

Tab. 17: Category F, backwards primer

No.	Primer sequence
88	GCA ACA GTT CAG CAA AGT CCA T

Tab. 18: Probes, category F

No.	Probe sequence	Probe pair
89	CGG TGA AGC CAC CGA CAT CGT	1
90	TGG CAG GTT CCG GCC TTC ACT CTC	1
91	AAGCCACCGACATCGTG	2
92	AAGCCACTGACATCGTG	2

The detection of the amplicons can take place through gel electrophoresis and detection of the DNA bands. Alternatively, the amplicons can be detected and quantified with the aid of probes. There are various ways of modifying probes to render a direct or indirect visual indication possible. They can be coupled to an anchor molecule which serves as

Duly registered and sworn translator Brigitte Ch. Müller

a linker. This type of anchor molecule may be, for example, a protein which is recognised by an antibody. This antibody may be coupled to an enzyme which causes a colour reaction, whereby the detection is provided. Peroxidase or catalase, for example, are used for these purposes. In addition, a probe can also be radioactively marked, whereby the measurement of the radioactivity leads to the detection and quantification.

Another way is to couple a fluorescent molecule to the probe. In this case it must be ensured that the fluorescence is only emitted or detected when the probe is bound to a single strand of the amplicon. This can be achieved in that the probe-amplicon hybrid is separated from the remaining PCR mixture. For example, probes can be bound to solid surfaces which "trap" the single-strand amplicons, whereby free probes are washed off.

On-line detection of the PCR products presents an elegant method. In this case, a fluorescence signal is only produced when a fluorescence-marked probe settles on an amplicon. This can occur in that the probe part of the amplicon-probe hybrid is selectively enzymatically broken down. Also, due to the opening up of the probe when it binds itself to the amplicon, quenching of the fluorescence signal is cancelled.

A further possibility is that two fluorescence-marked probes are used. It is only when both bind adjacently to an amplicon that a so-called FRET (Fluorescence Resonance Energy Transfer) can produce a signal (Fig. 1). This method has the substantial advantage that several specificity levels are a constituent part of the detection: firstly the primers bind to a certain target molecule, secondly both probes must bind to the "correct" amplicon and thirdly, they must be located adjacently in the correct order. With this adjacent arrangement the distance between the probes is decisive for the successful emission of the signal. Each of these requirements contributes to the increase in the specificity of the detection.

Alternatively, there are also fluorescence molecules which interact with the DNA double helix and then emit a signal. This unspecific detection of PCR products has however the disadvantage that erroneous amplification products are also detected.

According to the above description, the execution of the investigation requires a large number of components. Therefore, it is especially advantageous to offer them in one or more packages of a kit. Such a kit can also contain the reagents and chemicals for enriching the bacteria, the components for the DNA release and purification as well as the consumable material for carrying out the PCR and for the detection.

Description of the figures

Figure 1 shows the FRET principle schematically.

Figure 2 shows PCR products with primers of category D.

Figure 3 shows PCR products with primers of category E.

Figure 4 shows the amplification and real-time detection of the SltI and SltII genes for EHEC strains.

Figure 5 shows the amplification and real-time detection of the eae gene for EHEC strains in a multiplex PCR reaction together with the Slt genes.

The following examples explain the invention

The illustrated Figures 1 – 5 were produced under the following conditions:

Figure 1: The schematic process of the FRET is shown. Numerous combinations of donor and acceptor are available. However, it is important that the absorption spectrum of the acceptor overlaps with the emission spectrum of the donor.

Only then is it ensured that excitation of the donor also leads to an adequately strong fluorescence with the acceptor.

Figure 2: Detection of EHEC with primers of category D. The test conditions largely correspond to those in the chapter "Detection of EHEC strains by PCR". The detection in the agarose gel also occurs as described in the above chapter.

Figure 3: Detection of EHEC with primers of category E. The test conditions largely correspond to those in the chapter "Detection of EHEC strains by PCR". The detection in the agarose gel also occurs as described in the above chapter.

Figure 4: This shows the amplification of SltI and SltII genes by real-time PCR. Probes are used which permit the detection of the SltI and also the SltII genes. These were coupled with the same fluorescence colouring agents (Lightcycler RED 640 and Fluorescein) so that the detection only occurs in one channel (F2). It can be seen that with the amplification of the SltII genes, signal curves with amplitudes arise which are larger than 14. The signal curves of the SltI genes lie significantly lower. If SltI and SltII both occur, then the amplitude exhibits the highest level. It is therefore an indicator for the occurrence and the differentiation between SltI and SltII genes.

From Figure 4 it can also be seen that depending on the application of the various probes, the signal amplitude for the SltI genes is of different heights. For the experiment shown, the primers nos. 1+6 and nos. 18+22 as well as the probes nos. 9+10 (for strain no. 1-10), probes nos. 95+96 (for strain nos. 11-20), probes nos. 97+98 (for strain nos. 21-30) and probes nos. 34+35 (for strains 1-30) were used. The probes were coupled with the colouring agents Fluorescein and Lightcycler Red 640. The detection occurred at a light wavelength of 640 nm.

It can be seen that the probes nos. 97+98 for strains, which only possess the Slt1 gene (see the table on page 56), produce the highest amplitude. This probe-primer combination is therefore especially well suited for on-line PCR.

Detection of the Slt genes: 25 (Slt2 without eae)// 5, 15 (Slt2 without eae), 3, 4 (Slt2+eae)// 2 (Slt2 without eae) 13, 14 (Slt2+eae)// 23 (Slt2+eae)// 24 (Slt2+eae)// 22 (Slt2 without eae)// 12 (Slt2 without eae)// 28, 29, 30 (Slt1+eae), 27 (Slt1+eae), 26 (Slt1+Slt2+eae)// 6, 16 (Slt1+Slt2+eae), 7, 8, 9, 10, 17, 18, 19, 20 (Slt1+eae)// 1, 11, 21 (water).

Figure 5 This shows the amplification and real-time detection of the eae genes for EHEC strains in a multiplex PCR with the Slt genes (Fig. 4).

The multiplex reaction was carried out together with the probes and primers from Fig. 4. For the detection of the eae gene the primers nos. 68+73 and the probes nos. 93+94 were used. The probes nos. 93+94 were coupled with the colouring agents Fluorescein and Lightcycler Red 705. The detection occurred at a light wavelength of 710 nm.

Two groups of curves can be seen. The curves with amplitudes >5 show a positive result for the eae gene. In this respect, strains are involved which possess an eae gene (see legend in Fig. 4, table page 56). The curves with amplitudes <5 indicate a negative result (water samples or strains without the eae gene (samples 1, 11, 21, 5, 15, 25).

<u>Detection of VTEC strains by PCR</u>

This invention is suitable for the detection of VTEC strains by the polymerase chain reaction. Referred to the complete genome, VTEC strains differ only slightly from conventional *E. coli* strains. For this reason it is not easy to identify the DNA or RNA sequences which unambiguously map the VTEC group. Since VTEC also exhibits

differences within itself, e.g. in the serotypes, a single sequence feature is not suitable for supplying an unambiguous detection.

The invention is based on a combination of several genotypical features being used for the detection, partly simultaneously and where necessary, partly consecutively. In addition primers and probes are provided which exploit the advantages of the PCR for the amplification and detection of the VTEC strains.

Detection of the VTEC strains can occur in various steps, comprising bacterial enrichment, DNA/RNA release and isolation, PCR and (possibly simultaneously) detection of the amplicons.

For enrichment, the bacteria are shaken overnight in 2 ml of LB medium (10 g Bacto Tryptone, 5 g yeast extract, 10 g NaCl in 1 l of water) at 37°C. The bacterial culture was then spun off in a centrifuge at 10000 xg and resuspended in 100 μl of water. Then 50 μl 100 mM NaOH were added. The cells were lysated after 5 min. Following this, the solution was neutralised with 100 μl of 0.5 M Tris pH 8. Then the suspension was spun for 10 min. at 10000 xg in a centrifuge to remove insoluble constituents. Of this solution 1 μl was used in each case in the PCR reactions.

The PCR reaction was prepared as follows:

Sample volume – 1 μl
10 x PCR buffer – 2.5 μl
10 mM dNTP – 0.25 μl
10 μM forwards primer
Category A – 0.2 μl
10 μM backwards primer
Category A – 0.2 μl
10 μM forwards primer
Category B – 0.2 μl
10 μM backwards primer
Category B – 0.2 μl
10 μM forwards primer
Category C – 0.2 μl
10 μM backwards primer
Category C – 0.2 μl
50 mM $MgCl_2$ – 0.75 μl
5 U/μl Taq polymerase – 0.3 μl
Water – add. 25 μl

The above reaction mixture was firmly closed in 200 μl reaction vessels and incubated according to the following protocol in a PCR device.

95°C – 5 min.

92°C – 1 min.)
52°C – 1 min. x 35)
72°C – 0.5 min.)

72°C – 5 min.

In the reaction mixture one forwards and one backwards primer of the categories A, B, C (Tab. 1-9) were used in each case. For example, amplicons for the strains listed in the following table were produced with the primers nos. 1, 6, 18, 22, 26 and 30. Positive results were present for these strains, classified serologically as VTEC, because in each case bands produced by the PCR could be seen in the ethidium-bromide coloured 1% agarose gel.

Tab.: Detection of VTEC strains with the primers of categories A-C

	Strain no. (Biotecon Diagnostics)	**VTEC serotype**	**Result positive (+) / negative (-)**
1	Bc 4734	O26:H11	+
2	Bc 4735	O157:H-	+
3	Bc 4736		+
4	Bc 4737		+
5	Bc 4738	O157:H7	+
6	Bc 4945	O26:H-	+
7	Bc 4946	O157:H7	+
8	Bc 4947	O111:H-	+
9	Bc 4948	O157:H	+
10	Bc 4949	O5	+
11	Bc 5643	O2:H5	+
12	Bc 5644	O128	+
13	Bc 5645	O55:H-	+

	Strain no. (Biotecon Diagnostics)	VTEC serotype	Result positive (+) / negative (-)
14	Bc 5646	O69:H-	+
15	Bc 5647	O101:H9	+
16	Bc 5648	O103:'H2	+
17	Bc 5850	O22:H8	+
18	Bc 5851	O55:H-	+
19	Bc 5852	O48:H21	+
20	Bc 5853	O26:H11	+
21	Bc 5854	O157:H7	+
22	Bc 5855	O157:H-	+
23	Bc 5856	O26:H-	+
24	Bc 5857	O103:H2	+
25	Bc 5858	O26:H11	+
26	Bc 7832		+
27	Bc 7833	O Rough:H-	+
28	Bc 7834	ONT:H-	+
29	Bc 7835	O103:H2	+
30	Bc 7836	O57:H-	+
31	Bc 7837	ONT:H-	+
32	Bc 7838		+
33	Bc 7839	O128:H2	+
34	Bc 7840	O157:H-	+
35	Bc 7841	O23:H-	+
36	Bc 7842	O157:H-	+
37	Bc 7843		+
38	Bc 7844	O157:H-	+
39	Bc 7845	O103:H2	+
40	Bc 7846	O26:H11	+
41	Bc 7847	O145:H-	+

	Strain no. (Biotecon Diagnostics)	VTEC serotype	Result positive (+) / negative (-)
42	Bc 7848	O157:H-	+
43	Bc 7849	O156:H47	+
44	Bc 7850		+
45	Bc 7851	O157:H-	+
46	Bc 7852	O157:H-	+
47	Bc 7853	O5:H-	+
48	Bc 7854	O157:H7	+
49	Bc 7855	O157:H7	+
50	Bc 7856	O26:H-	+
51	Bc 7857		+
52	Bc 7858		+
53	Bc 7859	ONT:H-	+
54	Bc 7860	O129:H-	+
55	Bc 7861		+
56	Bc 7862	O103:H2	+
57	Bc 7863		+
58	Bc 7864	O Rough:H-	+
59	Bc 7865		+
60	Bc 7866	O26:H-	+
61	Bc 7867	O Rough:H-	+
62	Bc 7868		+
63	Bc 7869	ONT:H-	+
64	Bc 7870	O113:H-	+
65	Bc 7871	ONT:H-	+
66	Bc 7872	ONT:H-	+
67	Bc 7873		+

	Strain no. (Biotecon Diagnostics)	VTEC serotype	Result positive (+) / negative (-)
68	Bc 7874	O Rough:H-	+
69	Bc 7875	O157:H-	+
70	Bc 7876	O111:H-	+
71	Bc 7877	O146:H21	+
72	Bc 7878	O145:H-	+
73	Bc 7879	O22:H8	+
74	Bc 7880	O Rough:H-	+
75	Bc 7881	O145:H-	+
76	Bc 8275	O157:H7	+
77	Bc 8318	O55:K-:H-	+
78	Bc 8325	O157:H7	+
79	Bc 8333		+
80	Bc 8332	ONT	+
81	Bc 5580	O157:H7	+
82	Bc 5582	O3:H	+
83	Bc 5579	O157:H7	+

In addition the amplicons could be detected with fluorescence-marked probe pairs from the categories A, B and C, that is, for example, with the probes SEQ ID no. 9, 10, 34, 35, 95, 96, 97, 98 and 40 + 41.

Detection of EHEC strains by PCR

Enterohemorrhagic *E. coli* can cause severe diarrhoea illnesses as germs contaminating foodstuffs. They are responsible for the HUS (haemolytic-uraemic syndrome), characterised by blood-containing diarrhoea and acute kidney failure. The illness can be fatal.

The EHEC can systematically be regarded as a subgroup of the VTEC. For this reason the detection can occur in two stages in which firstly the VTEC are detected according to Example 1 and then the EHEC detection occurs from the positive findings.

In this example strains in the following table are examined:

No.	Biotecon No.	Sero var.	VTEC +/-	EHEC +/-
1	BC 12503	O157H-	+	+
2	BC 12507	O157H-	+	+
3	BC 12408	O84H21	+	+
4	BC 12518	O157H7	+	+
5	BC 12530	O156H-	+	+
6	BC 12538	O157H7	+	+
7	BC 12543	O111H-	+	+
8	BC 12544	O26H11	+	+
9	BC 12545	O103H2	+	+
10	BC 12546	O118H-	+	+
11	BC 12547	O118H-	+	+

The detection of the EHEC strains can occur in various steps, comprising bacterial enrichment, DNA/RNA release and isolation, PCR and (possibly simultaneously) detection of the amplicons.

For enrichment the bacteria are shaken overnight in 2 ml LB medium (10 g Bacto Tryptone, 5 g yeast extract, 10 g NaCl in 1 l of water) at 37°C. The bacterial culture was then spun off in a centrifuge at 10000 xg and resuspended in 100 μl of water. Then 50 μl 100 mM NaOH were added. The cells were lysated after 5 min. Following this, the solution was neutralised with 100 μl of 0.5 M Tris pH 8. Then the suspension was spun for 10 min. at 10000 xg in a centrifuge to remove insoluble constituents. Of this solution 1 μl was used in each case in the PCR reactions.

The PCR reaction was prepared as follows:

Sample volume – 1 µl
10 x PCR buffer – 2.5 µl
10 mM dNTP – 0.25 µl
10 µM forwards primer
Category A – 0.2 µl
10 µM backwards primer
Category A – 0.2 µl
10 µM forwards primer
Category B – 0.2 µl
10 µM backwards primer
Category B – 0.2 µl
10 µM forwards primer
Category C – 0.2 µl
10 µM backwards primer
Category C – 0.2 µl
50 mM $MgCl_2$ – 0.75 µl
5 U/µl Taq polymerase – 0.3 µl
Water – add. 25 µl

The above reaction mixture was firmly closed in 200 µl reaction vessels and incubated according to the following protocol in a PCR device.

95°C – 5 min.

92°C – 1 min.)
52°C – 1 min. x 35)
72°C – 0.5 min.)

72°C – 5 min.

In the reaction mixture one forwards and one backwards primer of the categories A, B, C (Tab. 1-9) were used in each case. For example, amplicons were produced with the primers nos. 1, 6, 18, 22, 26 and 30. Positive results were present for these strains, classified serologically as EHEC, because in each case bands produced by the PCR could be seen in the ethidium-bromide coloured 1% agarose gel.

The DNA of the positive results was again examined in a second run. In this run a PCR with forwards and backwards primers of the categories D and E is used. The following protocol is used:

Sample volume – 1 μl
10 x PCR buffer – 2.5 μl
10 mM dNTP – 0.25 μl
10 μM forwards primer
    Category C – 0.2 μl
10 μM backwards primer
    Category C – 0.2 μl
10 μM forwards primer
    Category D – 0.2 μl
10 μM backwards primer
    Category D – 0.2 μl
50 mM $MgCl_2$ – 0.75 μl
5 U/μl Taq polymerase – 0.3 μl
Water – add. 25 μl

The above reaction mixture was firmly closed in 200 μl reaction vessels and incubated according to the following protocol in a PCR device.

95°C – 5 min.

92°C – 1 min.)
52°C – 1 min. x 35)
72°C – 0.5 min.)

72°C – 5 min.

As a primer of category D, for example, the combination of primers nos. 46, 54 and nos. 68 and 73 can be used. It is also possible to use this primer pair in parallel PCR reactions. The results from two separate PCR runs are illustrated in the following.

Since the bands of Figures 2 and 3 have different sizes, they can also be detected in a gel, originating from a single PCR reaction, as double bands. Furthermore, the bands can be detected by the previously described FRET technology in that probe pairs of categories D and E are used. For example, the probes nos. 60, 61 and 78, 79 can be used for this purpose.

<u>Specificity of the EHEC detection</u>

As previously described, the EHEC detection preferably occurs in at least two steps, comprising PCR reactions with the primer categories A-C and D-E. Here, positive results from the first step are further examined in a second step. If the first step turns out to be negative, this result can be checked by an appropriate control in which *E. coli* is detected. Furthermore, it is important that the primers of categories A-C do not indicate any incorrect positive results. For this reason their specificity has been intensively examined. The results are presented in the following.

For enrichment the bacteria are shaken overnight in 2 ml LB medium (10 g Bacto Tryptone, 5 g yeast extract, 10 g NaCl in 1 l of water) at 37°C. The bacterial culture was then spun off in a centrifuge at 10000 xg and resuspended in 100 μl of water. Then 50 μl 100 mM NaOH were added. The cells were lysated after 5 min. Following this, the solution was neutralised with 100 μl of 0.5 M Tris pH 8. The suspension was then spun for 10 min. at 10000 xg in a centrifuge to remove insoluble constituents. Of this solution 1 μl was used in each case in the PCR reactions.

The PCR reaction was prepared as follows:

Sample volume – 1 μl
10 x PCR buffer – 2.5 μl
10 mM dNTP – 0.25 μl
10 μM forwards primer
    Category A – 0.2 μl
10 μM backwards primer
    Category A – 0.2 μl
10 μM forwards primer
    Category B – 0.2 μl
10 μM backwards primer
    Category B – 0.2 μl
10 μM forwards primer
    Category C – 0.2 μl
10 μM backwards primer
    Category C – 0.2 μl
50 mM $MgCl_2$ – 0.75 μl
5 U/μl Taq polymerase – 0.3 μl
Water – add. 25 μl

The above reaction mixture was firmly closed in 200 μl reaction vessels and incubated according to the following protocol in a PCR device.

95°C – 5 min.

92°C – 1 min.)
52°C – 1 min. x 35)
72°C – 0.5 min.)

72°C – 5 min.

One forwards and one backwards primer of the categories A, B, C (Tab. 1-9) in each case was used in the reaction mixture. For example, with the primers nos. 1, 6, 18, 22, 26 and 30 no amplicons were produced with the strains listed in the following table. Negative results were consequently present for these strains, because in no case could bands of the expected size produced by the PCR be seen in the ethidium-bromide coloured 1% agarose gel. Since the correct DNA fragments were not amplified, also no incorrect positive result can arise due to probes of the categories A-C. This, too, was experimentally verified.

Tab.: Bacterial strains tested as negative controls

Species	Strain no.	PCR detection
Aeromonas hydrophila	DSM 30188	-
Pseudomonas cepacia	BC 3134	-
Pseudomonas paucimobilis	DSM 1098	-
Lactobacillus bifermentans	BC 8463	-
Flavobacterium johnsonii	DSM 2064	-
Flavobacterium flavense	DSM 1076	-
Flavobacterium resinovorum	DSM 7478	-
Enterococcus casseliflavus	BC 7629	-
Comamonas testosteroni	BC 4276	-
Alcaligenes latus	DSM 1122	-
Budvicia aquatica	BC 8923	-

Species	Strain no.	PCR detection
Achromobacter ruhlandii	BC 8908	-
Achromobacter xylosa	BC 8913	-
Sphingobacterium multivorans	BC 8924	-
Ralstonia pickettii	BC 5368	-
Sphingomonas paucimobilis	BC 5293	-
Acinetobacter calcoaceticus	DSM 590	-
Aeromonas hydrophila	DSM 6173	-
Aeromonas enteropeloges	DSM 6394	-
Moraxella catarrhalis	DSM 9143	-
Pasteurella pneumotropica	DSM 2891	-
Pseudomonas beijerinkii	DSM 7218	-
Stenotrophomonas putrefaciens	BC 5337	-
Xanthomonas maltophila	BC 4273	-
Brochotrix thermosphacta	DSM 20171	-
Brochotrix thermophilus	DSM 20594	-
Brochotrix campestris	DSM 4712	-
Staphylococcus haemolyticus	BC 2747	-
Staphylococcus chromogenes	BC 5468	-
Staphylococcus gallinosum	BC 5472	-
Staphylococcus lentus	BC 5462	-
Staphylococcus intermedius	DSM 20036	-
Staphylococcus saprophyticus	DSM 20038	-
Staphylococcus hominis	BC 5466	-
Staphylococcus equorum	BC 9447	-
Staphylococcus sciuri	BC 5461	-
Staphylococcus hyicus	BC 5469	-
Aeromonas caviae	DSM 7326	-
Pantoea stewartii	DSM 30176	-
Xhenorhabdus poinarii	DSM 4768	-
Klebsiella ornitholytica	DSM 7464	-
Vibrio vulnificus	DSM 10147	-
Moellerella wisconsis	DSM 5079	-
Yersinia pseudotuberculosis	BC 8723	-
Vibrio mimicus	DSM 33653	-
Aeromonas sobriae	ATCC 43979	-
Pasteurella aerogenes	DSM 10153	-
Listonella anguillarum	DSM 11323	-

Use of *E. coli* positive controls

As described previously, EHEC strains are detected according to the invention in two steps by using the primers A-C and D-E. If the PCR reactions of the first step indicate a positive result, the samples are examined further in a second step. If on the other hand Step 1 turns out to be negative, then there is no VTEC and therefore also no EHEC strain present. However, it must be ensured that experimental errors can be eliminated. One possibility involves the detection of *E. coli*, because this germ is present in almost all foodstuffs relevant to EHEC. By doping a foodstuff with an *E. coli* strain there is the possibility of using this harmless control germ on a routine basis. In addition detection of *E. coli* is often desired from a hygiene point of view.

From pure cultures of the bacteria listed in the following table genomic DNA was isolated using a familiar standard method. Approximately 1 to 10 ng of each of these preparations were then used in the presence of each of 0.4 μM of an equimolar oligonucleotide mixture nos. 84-87 and 0.4 μM oligonucleotide no. 88, 2 mM $MgCl_2$, 200 μM dNTP (Roche Diagnostics, dUTP was used instead of dTTP), and 0.03 U/μl Taq polymerase (Life Technologies) in a single concentrated reaction buffer (Life Technologies) in the PCR. The PCR was carried out in a Perkin Elmer 9600 Thermocycler with the following listed thermal profile:

Initial denaturing	95°C	5 min.
Amplification (35 cycles)	95°C	20 s.
	63°C	45 s.
Final synthesis	72°C	5 min.

After termination of the PCR reaction the amplification products were fractionated using agarose-gel electrophoresis and rendered visible by colouration with ethidium bromide. The expected products of a length of 351 base pairs where only observed in the cases in which DNA of strains of the species *E. coli* or the genus *Shigella* was present. The DNA fractionated in the gels was transferred to nylon filters in a familiar standard

method and hybridised for checking the specificity with the oligonucleotides nos. 91 and 92 marked on the 5' end with biotin. The hybridisation occurred in 5 x SSC, 2% blocking reagent, 0.1% lauroyl sarcosine, 0.02% SDS and 5 pmol/ml of probe for 4 hrs at 52°C. Washing took place in 2 x SSC, 0.1% SDS for 2 x 10 min. at 52°C. The detection occurred in a familiar standard method using alkaline phosphatase conjugates (ExtrAvidin, Sigma) in the presence of 5-bromo-4-chloro-3-indolyl phosphate and 4-nitro blue tetrazolium chloride (Boehringer Mannheim). On the filters a band was observed only in those cases in which a band of 351 base pairs were previously visible on the agarose gel. Hence, the presence of all 645 tested *E. coli* and 32 *Shigella* strains was detected (see following table) using PCR and hybridisation. In contrast, none of the tested bacterial strains not belonging to this species was acquired with this system.

Table: List of the tested bacteria of the *E. coli/Shigella* group

Species	**Strain no.**	**Serotype**	**Pathotype**	**PCR detection**	**Hybridisation with probes**
E. coli	NCTC 12757	n.d.		+	+
E. coli	NCTC 12779	n.d.		+	+
E. coli	NCTC 12790	n.d.		+	+
E. coli	NCTC 12796	n.d.		+	+
E. coli	NCTC 12811	n.d.		+	+
E. coli	ATCC 11229	n.d.		+	+
E. coli	ATCC 25922	n.d.		+	+
E. coli	ATCC 8739	n.d.		+	+
E. coli	DSM 30083	O1:K1:H7		+	+
E. coli	BC 5849	O111:H2		+	+
E. coli	BC 8265	O104		+	+
E. coli	BC 8267	O55		+	+
E. coli	BC 8268	O6:H16		+	+
E. coli	BC 8270	O55:K(59):H-		+	+
E. coli	BC 8271	O55		+	+
E. coli	BC 8272	O55:K-:H-		+	+
E. coli	BC 8273	O55		+	+
E. coli	BC 8276	O128:K-H-		+	+
E. coli	BC 8277	O128:K68:H2		+	+
E. coli	BC 8278	O126		+	+
E. coli	BC 8279	O126		+	+
E. coli	BC 8312	ONT:H-		+	+

Species	Strain no.	Serotype	Pathotype	PCR detection	Hybridisation with probes
E. coli	BC 8317	O158:K-:H23		+	+
E. coli	BC 8319	O128:H21		+	+
E. coli	BC 8320	O55:H-		+	+
E. coli	BC 8321	O55		+	+
E. coli	BC 8322	O55		+	+
E. coli	BC 8326	O104		+	+
E. coli	BC 8327	037		+	+
E. coli	BC 8331	O24		+	+
E. coli	BC 8335	O119:H27		+	+
E. coli	BC 8338	O10:H4		+	+
E. coli	BC 8341	O110:H17		+	+
E. coli	BC 8344	O103		+	+
E. coli	BC 8345	O103		+	+
E. coli	BC 8346	O44		+	+
E. coli	BC 8347	O44		+	+
E. coli	BC 8348	O44		+	+
E. coli	BC 8863	n.d.		+	+
E. coli	BC 8864	n.d.		+	+
E. coli	BC 4734	O26:H11	VTEC	+	+
E. coli	BC 4735	O157:H-	VTEC	+	+
E. coli	BC 4736	n.d.	VTEC	+	+
E. coli	BC 4737	n.d.	VTEC	+	+
E. coli	BC 4738	O157:H7	VTEC	+	+
E. coli	BC 4945	O26:H-	VTEC	+	+
E. coli	BC 4946	O157:H7	VTEC	+	+
E. coli	BC 4947	O111:H-	VTEC	+	+
E. coli	BC 4948	O157:H	VTEC	+	+
E. coli	BC 4949	O5	VTEC	+	+
E. coli	BC 5579	O157:H7	VTEC	+	+
E. coli	BC 5580	O157:H7	VTEC	+	+
E. coli	BC 5582	O3:H	VTEC	+	+
E. coli	BC 5643	O2:H5	VTEC	+	+
E. coli	BC 5644	O128	VTEC	+	+
E. coli	BC 5645	O55:H-	VTEC	+	+
E. coli	BC 5646	O69:H-	VTEC	+	+
E. coli	BC 5647	O101:H9	VTEC	+	+
E. coli	BC 5648	O103:H2	VTEC	+	+
E. coli	BC 5850	O22:H8	VTEC	+	+
E. coli	BC 5851	O55:H-	VTEC	+	+
E. coli	BC 5852	O48:H21	VTEC	+	+
E. coli	BC 5853	O26:H11	VTEC	+	+
E. coli	BC 5854	O157:H7	VTEC	+	+
E. coli	BC 5855	O157:H-	VTEC	+	+
E. coli	BC 5856	O26:H-	VTEC	+	+
E. coli	BC 5857	O103:H2	VTEC	+	+

Species	Strain no.	Serotype	Pathotype	PCR detection	Hybridisation with probes
E. coli	BC 5858	O26:H11	VTEC	+	+
E. coli	BC 7832	n.d.	VTEC	+	+
E. coli	BC 7833	O Rough:H-	VTEC	+	+
E. coli	BC 7834	ONT:H-	VTEC	+	+
E. coli	BC 7835	O103:H2	VTEC	+	+
E. coli	BC 7836	O57:H-	VTEC	+	+
E. coli	BC 7837	ONT:H-	VTEC	+	+
E. coli	BC 7838	n.d.	VTEC	+	+
E. coli	BC 7839	O128:H2	VTEC	+	+
E. coli	BC 7840	O157:H-	VTEC	+	+
E. coli	BC 7841	O23:H-	VTEC	+	+
E. coli	BC 7842	O157:H-	VTEC	+	+
E. coli	BC 7843	n.d.	VTEC	+	+
E. coli	BC 7844	O157:H-	VTEC	+	+
E. coli	BC 7845	O103:H2	VTEC	+	+
E. coli	BC 7846	O26:H11	VTEC	+	+
E. coli	BC 7847	O145:H-	VTEC	+	+
E. coli	BC 7848	O157:H-	VTEC	+	+
E. coli	BC 7849	O156:H47	VTEC	+	+
E. coli	BC 7850	n.d.	VTEC	+	+
E. coli	BC 7851	O157:H-	VTEC	+	+
E. coli	BC 7852	O157:H-	VTEC	+	+
E. coli	BC 7853	O5:H-	VTEC	+	+
E. coli	BC 7854	O157:H7	VTEC	+	+
E. coli	BC 7855	O157:H7	VTEC	+	+
E. coli	BC 7856	O26:H-	VTEC	+	+
E. coli	BC 7857	n.d.	VTEC	+	+
E. coli	BC 7858	n.d.	VTEC	+	+
E. coli	BC 7859	ONT:H-	VTEC	+	+
E. coli	BC 7860	O129:H-	VTEC	+	+
E. coli	BC 7861	n.d.	VTEC	+	+
E. coli	BC 7862	O103:H2	VTEC	+	+
E. coli	BC 7863	n.d.	VTEC	+	+
E. coli	BC 7864	O Rough:H-	VTEC	+	+
E. coli	BC 7865	n.d.	VTEC	+	+
E. coli	BC 7866	O26:H-	VTEC	+	+
E. coli	BC 7867	O Rough:H-	VTEC	+	+
E. coli	BC 7868	n.d.	VTEC	+	+
E. coli	BC 7869	ONT:H-	VTEC	+	+
E. coli	BC 7870	O113:H-	VTEC	+	+
E. coli	BC 7871	ONT:H-	VTEC	+	+
E. coli	BC 7872	ONT:H-	VTEC	+	+
E. coli	BC 7873	n.d.	VTEC	+	+
E. coli	BC 7874	O Rough:H-	VTEC	+	+
E. coli	BC 7875	O157:H-	VTEC	+	+

Species	Strain no.	Serotype	Pathotype	PCR detection	Hybridisation with probes
E. coli	BC 7876	O111:H-	VTEC	+	+
E. coli	BC 7877	O146:H21	VTEC	+	+
E. coli	BC 7878	O145:H-	VTEC	+	+
E. coli	BC 7879	O22:H8	VTEC	+	+
E. coli	BC 7880	O Rough:H-	VTEC	+	+
E. coli	BC 7881	O145:H-	VTEC	+	+
E. coli	BC 8275	O157:H7	VTEC	+	+
E. coli	BC 8318	O55:K-:H-	VTEC	+	+
E. coli	BC 8325	O157:H7	VTEC	+	+
E. coli	BC 8332	ONT	VTEC	+	+
E. coli	BC 8333	n.d.	VTEC	+	+
E. coli	BC 8246	O152:K-:H-	EIEC	+	+
E. coli	BC 8247	O124:K(72):H3	EIEC	+	+
E. coli	BC 8248	O124	EIEC	+	+
E. coli	BC 8249	O112	EIEC	+	+
E. coli	BC 8250	O136:K(78):H-	EIEC	+	+
E. coli	BC 8251	O124:H-	EIEC	+	+
E. coli	BC 8252	O144:K-:H-	EIEC	+	+
E. coli	BC 8253	O143:K:H-	EIEC	+	+
E. coli	BC 8254	O143	EIEC	+	+
E. coli	BC 8255	O112	EIEC	+	+
E. coli	BC 8256	O28a.e	EIEC	+	+
E. coli	BC 8257	O124:H-	EIEC	+	+
E. coli	BC 8258	O143	EIEC	+	+
E. coli	BC 8259	O167:K-:H5	EIEC	+	+
E. coli	BC 8260	O128a.c.:H35	EIEC	+	+
E. coli	BC 8261	O164	EIEC	+	+
E. coli	BC 8262	O164:K-:H-	EIEC	+	+
E. coli	BC 8263	O164	EIEC	+	+
E. coli	BC 8264	O124	EIEC	+	+
E. coli	BC 7567	O86	EPEC	+	+
E. coli	BC 7568	O128	EPEC	+	+
E. coli	BC 7571	O114	EPEC	+	+
E. coli	BC 7572	O119	EPEC	+	+
E. coli	BC 7573	O125	EPEC	+	+
E. coli	BC 7574	O124	EPEC	+	+
E. coli	BC 7576	O127a	EPEC	+	+
E. coli	BC 7577	O126	EPEC	+	+
E. coli	BC 7578	O142	EPEC	+	+
E. coli	BC 7579	O26	EPEC	+	+
E. coli	BC 7580	OK26	EPEC	+	+
E. coli	BC 7581	O142	EPEC	+	+
E. coli	BC 7582	O55	EPEC	+	+
E. coli	BC 7583	O158	EPEC	+	+
E. coli	BC 7584	O-	EPEC	+	+

Species	Strain no.	Serotype	Pathotype	PCR detection	Hybridisation with probes
E. coli	BC 7585	O-	EPEC	+	+
E. coli	BC 7586	O-	EPEC	+	+
E. coli	BC 8330	n.d.	EPEC	+	+
E. coli	BC 8550	O26	EPEC	+	+
E. coli	BC 8551	O55	EPEC	+	+
E. coli	BC 8552	O158	EPEC	+	+
E. coli	BC 8553	O26	EPEC	+	+
E. coli	BC 8554	O158	EPEC	+	+
E. coli	BC 8555	O86	EPEC	+	+
E. coli	BC 8556	O128	EPEC	+	+
E. coli	BC 8557	OK26	EPEC	+	+
E. coli	BC 8558	O55	EPEC	+	+
E. coli	BC 8560	O158	EPEC	+	+
E. coli	BC 8561	O158	EPEC	+	+
E. coli	BC 8562	O114	EPEC	+	+
E. coli	BC 8563	O86	EPEC	+	+
E. coli	BC 8564	O128	EPEC	+	+
E. coli	BC 8565	O158	EPEC	+	+
E. coli	BC 8566	O158	EPEC	+	+
E. coli	BC 8567	O158	EPEC	+	+
E. coli	BC 8568	O111	EPEC	+	+
E. coli	BC 8569	O128	EPEC	+	+
E. coli	BC 8570	O114	EPEC	+	+
E. coli	BC 8571	O128	EPEC	+	+
E. coli	BC 8572	O128	EPEC	+	+
E. coli	BC 8573	O158	EPEC	+	+
E. coli	BC 8574	O158	EPEC	+	+
E. coli	BC 8575	O158	EPEC	+	+
E. coli	BC 8576	O158	EPEC	+	+
E. coli	BC 8577	O158	EPEC	+	+
E. coli	BC 8578	O158	EPEC	+	+
E. coli	BC 8581	O158	EPEC	+	+
E. coli	BC 8583	O128	EPEC	+	+
E. coli	BC 8584	O158	EPEC	+	+
E. coli	BC 8585	O128	EPEC	+	+
E. coli	BC 8586	O158	EPEC	+	+
E. coli	BC 8588	O26	EPEC	+	+
E. coli	BC 8589	O86	EPEC	+	+
E. coli	BC 8590	O127	EPEC	+	+
E. coli	BC 8591	O128	EPEC	+	+
E. coli	BC 8592	O114	EPEC	+	+
E. coli	BC 8593	O114	EPEC	+	+
E. coli	BC 8594	O114	EPEC	+	+
E. coli	BC 8595	O125	EPEC	+	+
E. coli	BC 8596	O158	EPEC	+	+

Species	Strain no.	Serotype	Pathotype	PCR detection	Hybridisation with probes
E. coli	BC 8597	O26	EPEC	+	+
E. coli	BC 8598	O26	EPEC	+	+
E. coli	BC 8599	O158	EPEC	+	+
E. coli	BC 8605	O158	EPEC	+	+
E. coli	BC 8606	O158	EPEC	+	+
E. coli	BC 8607	O158	EPEC	+	+
E. coli	BC 8608	O128	EPEC	+	+
E. coli	BC 8609	O55	EPEC	+	+
E. coli	BC 8610	O114	EPEC	(+)	+
E. coli	BC 8615	O158	EPEC	+	+
E. coli	BC 8616	O128	EPEC	+	+
E. coli	BC 8617	O26	EPEC	+	+
E. coli	BC 8618	O86	EPEC	+	+
E. coli	BC 8619	n.d.	EPEC	+	+
E. coli	BC 8620	n d.	EPEC	+	+
E. coli	BC 8621	n.d.	EPEC	+	+
E. coli	BC 8622	n.d.	EPEC	+	+
E. coli	BC 8623	n.d.	EPEC	+	+
E. coli	BC 8624	O158	EPEC	+	+
E. coli	BC 8625	O158	EPEC	+	+
E. coli	BC 5581	O78:H11	ETEC	+	+
E. coli	BC 5583	O2:K1	ETEC	+	+
E. coli	BC 8221	O118	ETEC	+	+
E. coli	BC 8222	O148:H-	ETEC	+	+
E. coli	BC 8223	O111	ETEC	+	+
E. coli	BC 8224	O110:H-	ETEC	+	+
E. coli	BC 8225	O148	ETEC	+	+
E. coli	BC 8226	O118	ETEC	+	+
E. coli	BC 8227	O25:H42	ETEC	+	+
E. coli	BC 8229	O6	ETEC	+	+
E. coli	BC 8231	O153:H45	ETEC	+	+
E. coli	BC 8232	O9	ETEC	+	+
E. coli	BC 8233	O148	ETEC	+	+
E. coli	BC 8234	O128	ETEC	+	+
E. coli	BC 8235	O118	ETEC	+	+
E. coli	BC 8237	O111	ETEC	+	+
E. coli	BC 8238	O110:H17	ETEC	+	+
E. coli	BC 8240	O148	ETEC	+	+
E. coli	BC 8241	O6H16	ETEC	+	+
E. coli	BC 8243	O153	ETEC	+	+
E. coli	BC 8244	O15:H-	ETEC	+	+
E. coli	BC 8245	O20	ETEC	+	+
E. coli	BC 8269	O125a.c:H-	ETEC	+	+
E. coli	BC 8313	O6:H6	ETEC	+	+
E. coli	BC 8315	O153:H-	ETEC	+	+

Species	Strain no.	Serotype	Pathotype	PCR detection	Hybridisation with probes
E. coli	BC 8329	n.d.	ETEC	+	+
E. coli	BC 8334	O118:H12	ETEC	+	+
E. coli	BC 8339	n.d.	ETEC	+	+

E. coli clinical isolates	359 (359)	359 (359)
E. coli food isolates	12 (12)	12 (12)
E. coli environmental isolates	23 (23)	23 (23)

Species	Strain no.	Serotype	Pathotype	PCR detection	Hybridisation with probes
Shigella boydii	DSM 7532	2		+	+
Sh. boydii	BC 7545	1		+	+
Sh. boydii	BC 7546	2		+	+
Sh. boydii	BC 7547	3		+	+
Sh. boydii	BC 7548	4		+	+
Sh. boydii	BC 7549	5		+	+
Sh. boydii	BC 7550	6		+	+
Sh. boydii	BC 7551	7		+	+
Sh. boydii	BC 7552	8		+	+
Sh. dysenteriae	NCTC 4837	1		+	+
Sh. dysenteriae	BC 7566	1		+	+
Sh. dysenteriae	BC 7553	2		+	+
Sh. dysenteriae	BC 7554	3		+	+
Sh. dysenteriae	BC 7555	5		+	+
Sh. dysenteriae	BC 7556	7		+	+
Sh. dysenteriae	BC 7557	8		+	+
Sh. dysenteriae	BC 7559	10		+	+
Sh. flexneri	DSM 4782	2a		+	+
Sh. flexneri	BC 5935	1a		+	+
Sh. flexneri	BC 5936	2a		+	+
Sh. flexneri	BC 5937	6		+	+
Sh. flexneri	BC 7560	1b		+	+
Sh. flexneri	BC 7561	2a		+	+
Sh. flexneri	BC 7562	3b		+	+
Sh. flexneri	BC 7563	4		+	+
Sh. flexneri	BC 7564	5		+	+
Sh. flexneri	BC 7565	6		+	+
Shigella sonnei	BC 1201			+	+
Shigella sonnei	BC 4302			+	+
Shigella sonnei	BC 4301			+	+
Shigella sonnei	BC 7889			+	+
Shigella sp.	BC 4303			+	+

ATCC: American Type Culture Collection (Manassas, USA)

BC: Strain Collection at BioteCon GmbH

DSM: German Collection of Micro-organisms (Braunschweig, Germany)

NCTC: National Collection of Type Cultures (London, United Kingdom)

+	= positive reaction	-	= negative reaction
(+)	= weak positive reaction	n.d.	= not determined

Table: List of the tested bacteria except the *E. coli/Shigella* group

Species	**Strain no.**	**PCR detection**	**Hybridisation with probes**
Buttiauxella agrestis	DSM 4586	-	-
Cedecea davisae	DSM 4568	-	-
Citrobacter amalonaticus	DSM 4593	-	-
Citrobactef freundii	DSM 30040	-	-
Citrobacter freundii	BC 6044	-	-
Citrobacter koseri	DSM 4570	-	-
Citrobacter koseri	DSM 4595	-	-
Citrobacter koseri	BC 4962	-	-
Edwartsiella tarda	DSM 30052	-	-
Enterobacter aerogenes	DSM 30053	-	-
Enterobacter aerogenes	BC 5895	-	-
Enterobacter amnigenus	DSM 4486	-	-
Enterobacter amnigenus	BC 7437	-	-
Enterobacter amnigenus	BC 8794	-	-
Enterobacter cloacae	DSM 30054	-	-
Enterobacter cloacae	BC 2467	-	-
Enterobacter cloacae	BC 8725	-	-
Enterobacter gergoviae	BC 511	-	-
Enterobacter gergoviae	BC 674	-	-
Enterobacter intermedius	DSM 4581	-	-
Enterobacter sakazakii	DSM 4485	-	-
Erwinia carotovora subsp. carotovora	DSM 30168	-	-
Escherichia blattae	NCTC 12127	-	-
Escherichia hermannii	DSM 4560	-	-
Escherichia hermannii	BC 8467	-	-
Escherichia fergusonii	NCTC 12128	(+)	-
Escherichia vulneris	DSM 4564	-	-
Escherichia vulneris	BC 8793	-	-
Hafnia alvei	BC 2154	-	-
Klebsiella oxytoca	DSM 5175	-	-

Species	Strain no.	PCR detection	Hybridisation with probes
Klebsiella oxytoca	BC 2468	-	-
Klebsiella planticola	DSM 4617	-	-
Klebsiella pneumoniae	BC 5365	-	-
Klebsiella pneumoniae subsp. pneumoniae	ATCC 13883	-	-
Klebsiella pneumoniae subsp. pneumoniae	DSM 30102	-	-
Klebsiella terrigena	DSM 2687	-	-
Kluyvera ascorbata	DSM 4611	-	-
Kluyvera sp.	BC 7440	-	-
Morganella morganii subsp. morganii	DSM 30164	-	-
Pantoea agglomerans	DSM 3493	-	-
Pantoea agglomerans	BC 6043	-	-
Pentoea agglomerans	BC 8600	-	-
Pantoea spp.	BC 8669	-	-
Pantoea spp.	BC 8726	-	-
Proteus mirabilis	DSM 788	-	-
Proteus rettgeri	DSM 1131	-	-
Providencia stuartii	DSM 4539	-	-
Rahnella aquatilis	DSM 4594	-	-
Salmonella bongori V	BC 5695	-	-
Salmonella bongori V	BC 7952	-	-
Salmonella enterica I	BC 7751	-	-
Salmonella enterica II	BC 5677	-	-
Salmonella enterica IIIa	BC 5241	-	-
Salmonella enterica IIIa	BC 5249	-	-
Salmonella enterica IIIb	BC 7937	-	-
Salmonella enterica IIIb	BC 7942	-	-
Salmonella enterica IV	BC 7759	-	-
Salmonella enterica VI	BC 7762	-	-
Serratia marcescens	BC 677	-	-
Serratia marcescens	DSM 1636	-	-
Serratia odorifera	BC 678	-	-
Serratia spp.	BC 1139	-	-
Yersinia enterocolytica	DSM 4780	-	-
Yersinia pseudotuberculosis	DSM 8992	-	-
Yokenella regensburgei	DSM 5079	-	-
Acinetobacter sp.	DSM 590	-	-
Aeromonas hydrophila subsp. hydrophila	DSM 6173	-	-
Bacillus cereus	NCFB 827	-	-
Bacillus stearothermophilus	DSM 1550	-	-
Bacillus subtilis	DSM 1970	-	-
Carnobacterium mobile	DSM 4848	-	-
Clostridium acetobutylicum	DSM 1731	-	-
Clostridium propionicum	DSM 1682	-	-
Clostridium saccharolyticum	DSM 2544	-	-
Comamonas testosteroni	DSM 1622	-	-

Species	**Strain no.**	**PCR detection**	**Hybridisation with probes**
Enterococcus faecalis	DSM 6134	-	-
Flavobacterum sp.	ATCC 27551	-	-
Haemophilus influenzae	DSM 4690	-	-
Lactococcus lactis subsp. hordniea	DSM 20450	-	-
Lactococcus raffinolactis	DSM 20443	-	-
Moraxella catarrhalis	DSM 9143	-	-
Pasteurella pneumotropica	BC 2891	-	-
Pediococcus inopinatus	DSM 20285	-	-
Pseudomonas aeruginosa	DSM 50071	-	-
Pseudomonas cepacia	BC 3134	-	-
Pseudomonas fluorescens	DSM 6290	-	-
Sphingomonas paucimobilis	BC 8795	-	-
Sphingomonas sp.	DSM 6014	-	-
Staphylococcus aureus subsp. aureus	DSM 20491	-	-
Stenotrophomonas maltophila	BC 8724	-	-
Streptococcus thermophilus	BC 2148	-	-
Vibrio alginolyticus	DSM 2171	-	-
Vibrio fischeri	DSM 507	-	-
Vibrio harveyi	DSM 6904	-	-
Vibrio parahaemolyticus	DSM 2172	-	-

Differentiation of Slt genes

A characteristic feature of the VTEC is the presence of one of the two genes SltI (Shiga-like toxin) or SltII or both genes. These genes are also known as vtx1 and vtx2. For the precise type classification of VTEC and EHEC strains, further differentiation can be made with regard to the presence of these genes or of variants of these genes. In this way important information for the propagation of these pathogenic *E. coli* strains and also for evolution can be obtained. In addition there are indications that the pathological potential for various SltI or SltII variants or for the occurrence of both genes varies.

For the differentiation between SltI and SltII genes the primers of category A or categories B+C can be used.

The PCR reaction I) was prepared as follows:

I)

Sample volume – 1 μl
10 x PCR buffer – 2.5 μl
10 mM dNTP – 0.25 μl
10 μM forwards primer
    Category A – 0.2 μl
10 μM backwards primer
    Category A – 0.2 μl
50 mM $MgCl_2$ – 0.75 μl
5 U/μl Taq polymerase – 0.3 μl
Water – add. 25 μl

The above reaction mixture was firmly closed in 200 μl reaction vessels and incubated according to the following protocol in a PCR device.

95°C – 5 min.

92°C – 1 min.)
52°C – 1 min. x 35)
72°C – 0.5 min.)

72°C – 5 min.

In the reaction mixture one forwards and one backwards primer of the category A (Tab. 1-9) was each used.

In a further PCR reaction II) the following mixture was prepared:

II)

Sample volume – 1 μl
10 x PCR buffer – 2.5 μl
10 mM dNTP – 0.25 μl
10 μM forwards primer
    Category B+C – 0.2 μl
10 μM backwards primer
    Category B+C – 0.2 μl
50 mM $MgCl_2$ – 0.75 μl
5 U/μl Taq polymerase – 0.3 μl
Water – add. 25 μl

The above reaction mixture was firmly closed in 200 μl reaction vessels and incubated according to the following protocol in a PCR device.

95°C – 5 min.

92°C – 1 min.)
52°C – 1 min. x 35)
72°C – 0.5 min.)

72°C – 5 min.

In the reaction mixture one forwards and one backwards primer of the categories B+C (Tab. 1-9) was each used.

The results of the PCR reactions are summarised in the following table. A positive result was obtained when an amplicon which produced a band in the magnitude of approx.

500-700 bp was amplified. This was rendered visible on an agarose gel coloured with ethidium bromide.

Table: Differentiation between SltI and SltII genes

				Genes present		PCR detection	
	BC no.	**Origin**	**Sero var.**	**SltI**	**SltII**	**Category A**	**Category B+C**
1	12502	Full-cream milk	O138H8	-	+	-	+
2	12503	Full-cream milk	O157H-	-	+	-	+
3	12504	Beef	O8H27	-	+	-	+
4	12505	Raw milk	O17H-	-	+	-	+
5	12506	Minced beef	O22H-	+	+	+	+
6	12507	Nuremberger grilled sausage	O157H-	-	+	-	+
7	12508	Lamb	O84H21	+	+	+	+
8	12509	Lamb	O7H-	+	+	+	+
9	12510	Lamb	OntH-	+	-	+	-
10	12511	Cheese from raw cow's milk	O23H15	-	+	-	+
11	12512	Minced beef, raw material	O8H-	-	+	-	+
12	12513	Minced beef, raw material	O- Rough H23	+	+	+	+
13	12514	Minced beef, raw material	O46H-	-	+	-	+
14	12515	Minced beef	O104H12	+	-	+	-
15	12516	Minced beef	O74H-	-	+	-	+
16	12517	Minced beef, raw material	O62H8	+	+	+	+
17	12518	Minced beef, raw material	O157H7	-	+	-	+
18	12519	Beef paté	O91H-	-	+	-	+
19	12520	Minced beef, raw material	O22H-	-	+	-	+
20	12521	Onion smoked sausage spread	O65H-	+	-	+	-
21	12522	Minced beef	O8H-	-	+	-	+
22	12523	Mixed minced meat	O91H21	+	+	+	+
23	12524	Minced beef, raw material	O113H4	-	+	-	+
24	12525	Minced beef	O22H8	+	+	+	+
25	12526	Minced beef, raw material	O113H4	+	+	+	+

The primers of the categories A resp. B+C are also to be used in order to amplify sub-types of the SltI (category A) and SltII (category B+C) genes as consensus primers. These sub-types can be differentiated with specific probes such as are listed for

categories A, resp. B+C. For sub-types not currently known, the probes of these categories can be tested empirically and assigned to the sub-types. Due to the large number of probes, a positive-negative pattern is produced which is characteristic of the sub-types. In addition, the primers of the categories A and B+C facilitate the amplification and subsequent sequencing of the amplicons. Also, techniques can be applied, such as mass spectrometry, hybridisation on biochips, "branch migration inhibition" or other techniques which enable an SNP (Single Nucleotide Polymorphism) analysis and are known to the specialist.

Optimisation of an on-line PCR

With an on-line PCR simultaneous amplification and detection of the amplicon occur. Depending on the amplicon to be detected, 1-2 colour-marked probes are added to the PCR mixture.

The detection of the amplicon can then take place, for example, with the aid of a 5' nuclease assay (TaqMan probes), using molecular beacons, Scorpion assays or the previously described FRET technology.

In particular in the latter case it can only be determined empirically which of the probe pairs to be used are optimally suited. Often, the obtained fluorescence signal is too weak to give a reliable and reproducible result. In addition, in a complex PCR mixture probes can form dimers with other probes or primers, so that no on-line detection occurs.

With the detection of EHEC it can be advantageous to amplify both the Slt genes (-> VTEC) as well as the eae genes in a single multiplex PCR reaction (Slt genes + eae gene = EHEC) and then also to detect them simultaneously. In this case very precise matching of the reaction components is required. Through the consumption of the nucleotides, the amplification of one of two DNA target regions can be prevented. This

signifies therefore that the amplification of a DNA is quenched by the amplification of another. It is therefore necessary to match all components of a PCR mixture to one another such that quenching does not occur.

This can also occur in that the primer concentration is limited. Here it must be considered that quenching is not a problem between the Slt genes, because the detection of only one Slt gene is adequate for the classification as VTEC. For this reason reduced amounts of SltI and SltII-specific primers can be added. The concentrations may be in the region of 300-200 nM per primer pair and PCR reaction. In contrast, the primer concentration of those for the eae gene should be higher (310-440 nM) in order to be able to also detect low eae DNA concentrations in the presence of higher Slt DNA concentrations.

A further method of preventing quenching due to the amplification of the Slt genes is to select an annealing temperature which is optimal for the eae-specific primers and less than optimal for the SltI and SltII-specific primers. Put more definitely, this temperature can be up to 5°C above the optimum temperature for all Slt primers. The thermodynamic melting point can be regarded as the optimum temperature for primers.

The methods of preventing quenching can be used reciprocally if eae genes are present in excess in relation to SltI and SltII genes or quench the Slt detection for other reasons.

In the following, PCR conditions are shown which enable simultaneous amplification of the Slt and eae genes.

The PCR reaction is prepared as follows:

Sample volume – 1 μl

10 x PCR buffer – 2 μl

Stabiliser – 5.53 μl

10 mM dNTP – 0.40 μl

10 – 4 μM forwards primer (primary sol.)

SEQ ID no. 1, 18, 68 – 0.2 μl

10 - 4 μM backwards primer

SEQ ID no. 6, 22, 73 – 0.2 μl

10 μM probes SEQ ID no. 93, 94, 95, 96, 97, 98, 9, 10, 35, 34

50 mM $MgCl_2$ – 1.6 μl

1 U/μl Taq polymerase – 1 μl

Water – add. 20 μl

Temperature cycles in the Lightcycler:

92°C – 0 min.)

57°C – 1 min. x 45)

72°C – 0.5 min.)

72°C – 5 min.

Figure 4 shows the amplification of SltI and SltII genes by real-time PCR. Probes were used which facilitate the detection both of the SltI and the SltII genes. These were each coupled with the same fluorescent colouring (Lightcycler RED 640 and Fluorescein), so that the detection occurred in one channel (F2) only. It can be seen that with the amplification of the SltII genes, signal curves arise with amplitudes greater than 14. The

signal curves of the SltI genes lie significantly lower. If both SltI and SltII genes occur, then the amplitude exhibits the highest level. It is therefore suitable as an indicator for the occurrence and the differentiation between the SltI and SltII genes.

It can also be seen from Figure 4 that, depending on the application of various probes, the signal amplitude for the SltI genes varies. In the illustration the probes nos. 9+10 (strain nos. 1-10), nos. 95+96 (strain nos. 11-20), nos. 97+98 (strain nos. 21-30) and probes nos. 34+35 (strain nos. 1-30) were used together with the primers nos. 1+6 and 18+22. In addition, the oligonucleotides for the detection of the eae genes (see below) are present in the PCR mixture.

The eae gene was detected with probes which are coupled with the fluorescent colourings Lightcycler RED 705 and Fluorescein. Their detection occurred therefore in a different channel (F3) than that used for the Slt genes (F2). The probes nos. 93+94 and the primers nos. 68+73 were used for the eae detection. It can be seen in Figure 5 that all eae-positive strains produce signal amplitudes which are greater than 5.

Table: Occurrence of pathogenicity genes with the VTEC/EHEC strains used in the real-time PCR

Strain no. in Figs. 4, 5	SltI	SltII	eae
2, 12, 22	-	+	+
3, 13, 23	-	+	+
4, 14, 24	+	+	-
5, 15, 25	-	+	-
6, 16, 26	+	-	+
7, 17, 27	+	-	+
8, 18, 28	+	-	+
9, 19, 29	+	-	+
10, 20, 30	+	-	+

Strains in the same row in the above table are each identical (e.g. 2=12=22).

As object of this invention, oligonucleotides are provided which are particularly well suited to the detection of EHEC or VTEC. Within the number of these oligonucleotides there are some which are particularly well suited for this detection. They are summarised in the following table.

Table: Preferred oligonucleotide combinations for the detection of pathogenic E. coli

Organisms to be detected	Primers	Probes
VTEC	No. 1+6+18+22	9+10, 95+96, 97+98, 34+35
VTEC	No. 1+6+18+22+84+85+86+87	9+10, 95+96, 97+98, 34+35, 89+90
EHEC (see Figs. 4+5)	No. 1+6+18+22, 68+73	9+10, 95+96, 97+98, 34+35, 93+94
EHEC	No. 1+6+18+22, 68+73+84+85+86+87	9+10, 95+96, 97+98, 34+35, 93+94, 89+90
EHEC	No. 1+6+18+22+46+54	9+10, 95+96, 97+98, 34+35, 60+61
EHEC	No. 1+6+18+22, 68+73+84+85+86+87+46+54	9+10, 95+96, 97+98, 34+35, 93+94, 89+90+60+61

Where a detection only occurs by visual indication of the amplicons in the agarose gel, the probes from the above table can be left out of the multiplex mixture.

Table: Optimisation of the real-time EHEC PCR

Problem	Solution
Specification as EHEC	Simultaneous amplification of the SltI/II genes and an eae gene or detection in two PCR steps, where necessary. Detection of the species Escherichia coli in addition to the pathogenicity genes.
Specification as EHEC	Simultaneous amplification of the SltI/II genes and of the hlyA gene or detection in two PCR steps, where necessary. Detection of the species Escherichia coli in addition to the pathogenicity genes.
Specification as EHEC	Simultaneous amplification of the SltI/II genes and of the eae gene and of the hlyA gene or detection in three PCR steps, where necessary. Detection of the species Escherichia coli in addition to the pathogenicity genes.
Various Slt genes are detected with the same fluorescent colouring	SltI and SltII genes can be differentiated by the curve traces and the height of the amplitude. Further differentiation possible through melting curve analysis.
The simultaneous amplification of the Slt and eae and/or hylA genes is quenched	Primers are limited.
The amplification of the Slt and eae and/or hlyA genes is quenched	Annealing temperatures of the primers and/or probes are optimally selected with regard to quenching.
The amplification of the Slt and eae and/or hlyA genes is quenched	Selection of the probes and primers reduces quenching significantly. The amplification efficiency is decisively influenced by these oligonucleotides. Therefore, the primers and probes were matched harmoniously with one another.
The signal level for probes is too low	Testing of a large number of probes/probe pairs and empirical selection of the best probes.

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Patent Claims

1. Method for the detection of EHEC bacteria in a sample, comprising the step:

   Detection of the occurrence of a nucleic acid sequence from the Slt locus in combination with a sequence from the eae locus and/or the hlyA locus in the sample.

2. Method according to Claim 1, characterised in that the detection includes at least one PCR.

3. Method according to one of the Claims 1 or 2, characterised in that for the detection at least one oligonucleotide is used comprising at least one sequence selected from one of the SEQ ID numbers 1 – 83 and 93 – 98 and derivatives of them.

4. Method according to one of the Claims 1 – 3, characterised in that at least one oligonucleotide is used comprising at least one sequence selected from one of the SEQ ID numbers 1 – 45 and 95 – 98 or derivatives of them (sequences of categories A – C) and at least one oligonucleotide comprising at least one sequence selected from one of the SEQ ID numbers 46 – 83 and 93 and 94 and derivatives of them (sequences of categories D and E).

5. Method according to one of the Claims 1 – 4, characterised in that a forwards primer with a backwards primer from one of the categories A – C is combined with a forwards primer and a backwards primer from one of the categories D and E.

6. Method according to one of the Claims 1 – 5, characterised in that an additional oligonucleotide is used comprising at least one sequence selected from one of the SEQ ID numbers 84 – 92 and derivatives of them (sequences of the category F).

7. Method according to one of the Claims 1 – 6, characterised in that several oligonucleotides are used in the scope of a multiplex PCR or in at least two separate sequential PCRs.

8. Method according to one of the Claims 1 – 7, characterised in that the detection includes bringing into contact the nucleic acid from the sample, after its amplification where necessary, with a biochip containing the oligonucleotides for the detection of EHEC.

9. Method according one of the Claims 1 – 8, characterised in that it comprises at least one further step selected from

    - amplification of the nucleic acid to be detected;
    - PCR amplification of the nucleic acid to be detected;
    - southern blot hybridisation of the nucleic acid to be detected with suitable probes, preferably selected from a nucleic acid comprising at least one sequence with one of the SEQ ID numbers 1- 98;
    - ligase chain reaction with the nucleic acid to be detected; and
    - isothermal nucleic acid amplification of the nucleic acid to be detected.

10. Method according to one of the Claims 1 – 9, characterised in that the detection comprises an on-line detection of obtained amplicons.

11. Method according to one of the Claims 1 – 10, characterised in that the amplification and/or detection of the nucleic acid to be detected occurs on a biochip.

12. Oligonucleotide for the detection of EHEC bacteria, selected from one of the nucleic acids comprising at least one sequence with one of the SEQ ID numbers 1 – 98 or derivatives of it.

13. Combination of oligonucleotides, comprising at least one oligonucleotide comprising at least one sequence selected from one of the categories A – C and at least one oligonucleotide comprising at least one sequence selected from one of the categories D and E, preferably one sequence D and one sequence from E.

14. Combination according to Claim 13, characterised in that it furthermore comprises an oligonucleotide comprising at least one sequence selected from the category F.

15. Kit for the detection of EHEC bacteria containing an oligonucleotide according to Claim 12 or a combination according to one of the Claims 13 or 14.

16. Application of an oligonucleotide according to Claim 12 and/or a combination according to Claim 13 or 14 for the detection of EHEC bacteria.

Application number / numéro de demande: EP01/ 11901

Figures: , 2 , 3

Pages:

Unscannable items
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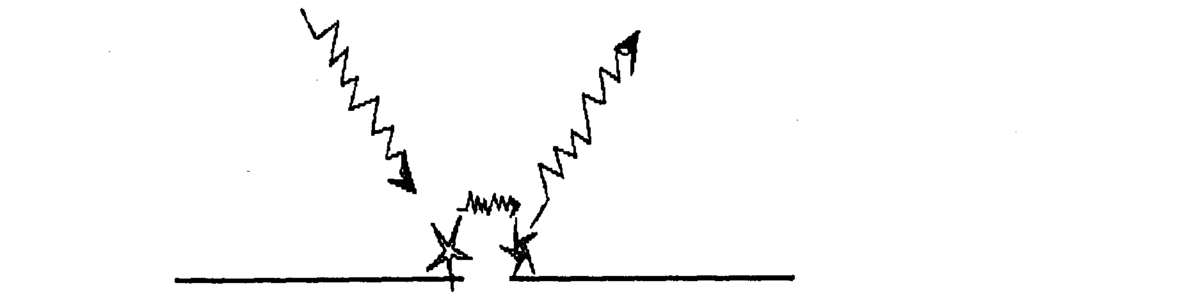
Documents reçu avec cette demande ne pouvant être balayés
(Commander les documents originaux dans la section de préparation des dossiers au 10ème étage)

Fig. 1



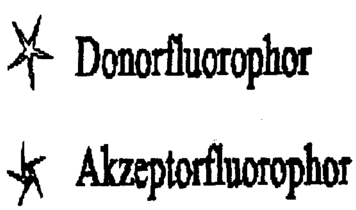


Fig. 1: Online-Detektion eines Amplikons durch FRET zwischen zwei Sonden

Fig. 1

* Donor fluorophor

* Acceptor fluorophor

Fig. 1: On-line detection of an amplicon by FRET between two probes.

Fig. 2

Fig. 2: Detection of sequence characteristics with primers of category D.

Fig. 3

Fig. 3: Detection of sequence characteristics with primers of category E.

FIG. 4

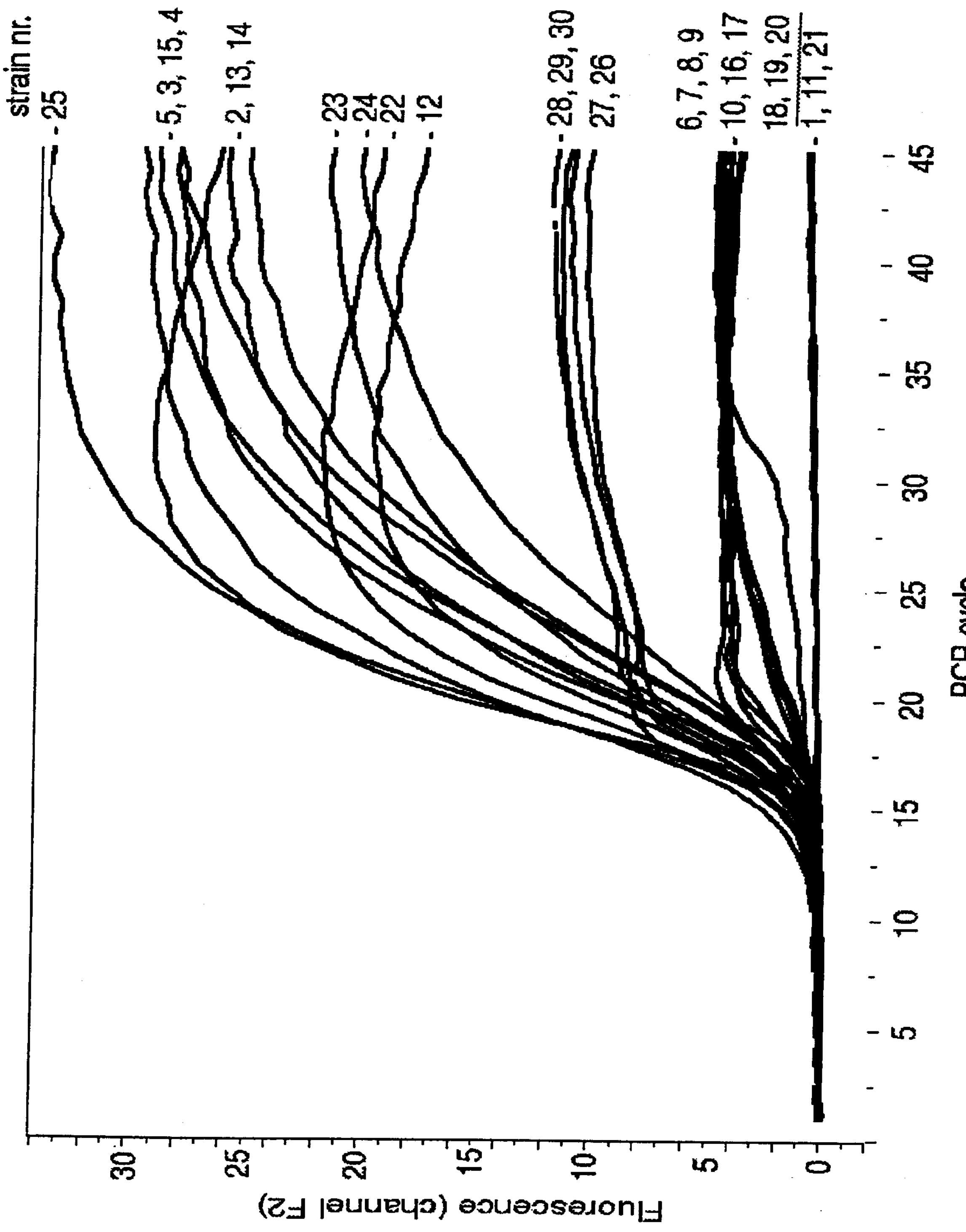


Fig. 4: Amplifikation und Realtime-Detektion der SltI- und SltII-Gene bei EHEC-Stämmen

Fig. 4

Strain nr. = Strain no.

Fig. 4: Amplification and real-time detection of the SltI and SltII genes for EHEC strains.

FIG. 5

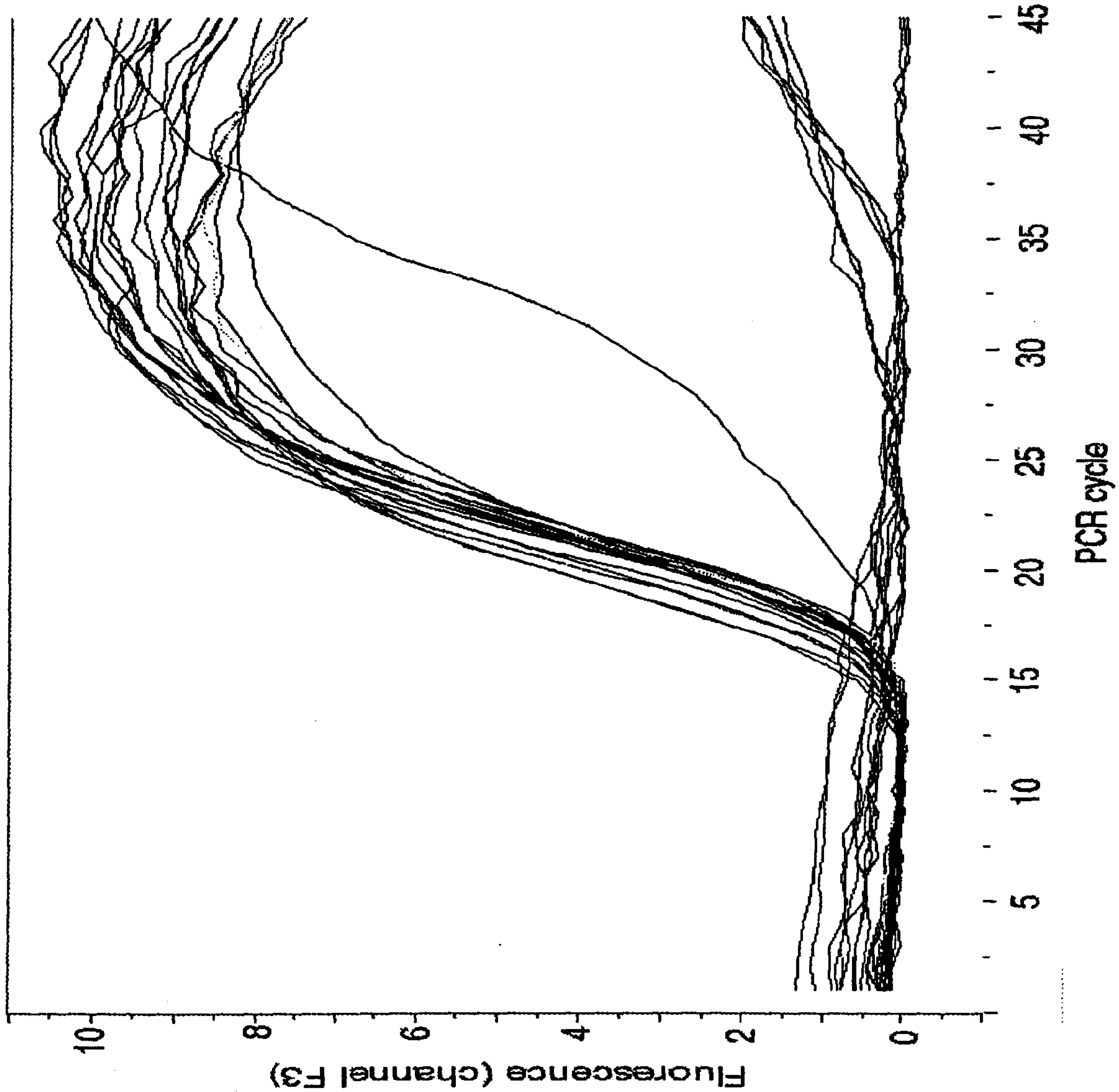


Fig. 5: Amplifikation und Realtime-Detektion der eae-Gene bei EHEC-Stämmen in einer Multiplex-PCR-Reaktion zusammen mit den Slt-Genen (Kanal F2)

Fig. 5

Fig. 5: Amplification and real-time detection of the eae genes for EHEC strains in a multiplex PCR reaction together with the Slt genes (Channel F2).